ANNUAL SCIENTIFIC REPORT

1991–1992

HOWARD HUGHES MEDICAL INSTITUTE

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*The primary purpose and objective of the
Howard Hughes Medical Institute shall be the promotion
of human knowledge within the field of basic
sciences (principally the field of medical research
and medical education) and the effective application
thereof for the benefit of mankind.*

—from the Charter
Incorporated December 17, 1953

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The Howard Hughes Medical Institute remains the largest private, nonprofit scientific and philanthropic organization in the United States engaged in the direct conduct of biomedical research. As an institute without walls, it conducts research in its laboratories headed by 222 investigators located at 53 medical schools, universities, and research institutions in 40 cities and 23 states across the nation. Through its philanthropic grants program, HHMI also supports various aspects of education in the sciences, from elementary school through postgraduate research training, and the research of outstanding biomedical scientists in selected countries outside of the United States. The total number of Institute employees, including 184 in its Bethesda offices, was 2,136. The budget in support of the research activities was $273.2 million and was $51.2 million in the grants program.

HHMI scientists conduct research in five broad areas that together encompass most of biomedical research: cell biology and regulation, genetics, immunology, neuroscience, and structural biology. In this volume, each investigator has reported the progress made by his or her research group during the past year. A bibliography citing the publications of each laboratory during this period is included. These chapters document a year of considerable achievements and progress toward the goals stated in the Institute's charter—"the promotion of human knowledge within the field of basic sciences (principally the field of medical research and medical education) and the effective application thereof for the benefit of mankind."

In recognition of the international nature of science, the Institute launched in 1991 a limited and experimental program whereby outstanding investigators, still in the developmental stages of their careers, were invited to apply for five-year research grants. The first countries selected were our neighbors, Canada and Mexico. You will read in this volume about the research accomplishments during the first grant year of the 24 scholars (14 from Canada and 10 from Mexico) initially selected. A second competition was held for scientists in Australia, New Zealand, and the United Kingdom. Grant awards are being made in 1993 to the institutions of the scholars selected in support of their work, and their reports will appear in later volumes. This international program is separate from the Institute's medical research program, in which HHMI investigators

are employees of the Institute. The international scholars do not join the HHMI staff; their research is supported by grants from the Institute.

Highlighting the year were six scientific meetings, one of which was attended by each of the Institute's investigators, the international scholars, and HHMI advisors. Most of the topics were selected to cut across the five program areas of the Institute and to bring together its scientists in different disciplines who otherwise might not meet to discuss their research. It has been gratifying to see the exchange of ideas and the development of collaborative associations during the sessions. The Institute also sponsors each year several workshops that explore in depth selected topics of special interest. These smaller, more informal gatherings are generally organized by one or several of the Institute's investigators or advisors and include also experts from outside the HHMI family and abroad. A list of these meetings is found elsewhere in this volume.

In 1988, through its grant programs, the Institute undertook the development of a number of activities to ensure that the scientific community will continue to be sustained and invigorated by the entrance of new talent and enthusiasm. The HHMI-supported programs in biomedical sciences now reach out to students at all levels, from kindergarten through postdoctoral research training. The institute supports graduate students, medical students, and physicians through fellowships; undergraduate institutions through an undergraduate science education grants program; and science museums through a public science and precollege education program. Details of these activities appear in this volume in the section on grants and special programs and in another annual HHMI publication, *Grants for Science Education*.

The Institute published the third in a series of occasional reports for a general audience on topics in basic biomedical sciences, *From Egg to Adult*. This joined two other reports, *Blazing a Genetic Trail* and *Finding the Critical Shapes*, which have been distributed free of charge to teachers, government officials, business leaders, journalists, and scientists, and are being used in over a thousand classrooms. Another publication aimed at providing an overview of the research of each of the Institute's investigators and international scholars in nontechnical language is the annual publication *Research in Progress*. A general *Annual Report of the How-*

ard Hughes Medical Institute describes the various programs for a lay audience and summarizes the Institute's financial data.

In the spring of 1991, construction began on the complex of buildings comprising the Institute's new headquarters and conference center in Chevy Chase, Maryland. The campus-like wooded grounds into which low brick buildings are set will provide a beautiful as well as functional home for the head- quarters staff and for the Institute's investigators when they gather to discuss their research. Dedication ceremonies at the site are planned for the spring of 1993, a year that also marks the Institute's forti- eth anniversary.

Purnell W. Choppin, M.D.
President

INTRODUCTION

This annual report summarizes the research conducted by the Institute's investigators from September 1, 1991, through August 31, 1992. At the end of August the Institute employed 222 investigators with supporting and administrative laboratory staffs numbering 1,674. Their research covers a diverse array of topics, although all fall within the scope of the five HHMI program areas of Cell Biology and Regulation, Genetics, Immunology, Neuroscience, and Structural Biology.

In addition this report includes the work of the Institute's first group of International Research Scholars. These 24 scientists from Canada and Mexico were selected in the Institute's international grants program that was launched in 1991 in recognition of the fact that scientific knowledge is not constrained by geographical or national boundaries. Five-year research grants were made to the scholars' institutions for the support of their research that is described in this volume. These reports cover the first year of grant support (July 1, 1991–March 30, 1992), and the publications listed have benefited directly from HHMI funding.

The 222 HHMI laboratories engaged in the direct conduct of medical research are directed by 4 Senior Investigators, 110 Investigators, 49 Associate Investigators, and 59 Assistant Investigators and assisted at the postdoctoral level by 29 Senior Associates and 546 Associates (including 11 who competed successfully in the Institute's research fellowship program for physicians and 7 who were identified through the Life Sciences Research Foundation but are employed by HHMI). The work of these scientists was aided by the efforts of a further 1,099 HHMI employees comprising 622 research specialists and research technicians, 210 administrative assistants and research secretaries, and 176 other support personnel, as well as by 91 individuals associated with the Institute's Offices of Administrative Services located within the Institute's medical school–based units. Also working within the Institute's laboratories and contributing to the work of the HHMI investigators, although not employed by the Institute, were a further 575 postdoctoral fellows and more than 800 graduate students.

The Institute's collaborative program with the National Institutes of Health at the Cloister on the NIH campus supported as employees 42 Research Scholars who were engaged in research within NIH laboratories under the direction of a number of senior scientists within the various Institutes of Health. The Research Scholar's program, directed for the Institute by Dr. Donald H. Harter, Senior Scientific Officer, has an administrative staff of 9.

LOCATIONS

The Institute maintained laboratories at 53 sites in 40 cities and 23 states, as listed elsewhere in this report. New laboratory facilities were completed at the University of California at Los Angeles and at the Rockefeller University. Construction was nearing completion for new structural biology laboratories at Yale University, and during the course of the year, several of the Institute's laboratories were either renovated or enlarged.

The Institute appointed 15 new investigators during the year at institutions where there were existing collaborative agreements; 13 other investigators were promoted within the Institute. The new investigators are Investigator Larry Simpson, Ph.D., at the University of California at Los Angeles; Associate Investigators Michael P. Bevilacqua, M.D., Ph.D., and Scott D. Emr, Ph.D., at the University of California at San Diego; Investigators Peter Cresswell, Ph.D., and Pietro De Camilli, M.D., Associate Investigator Reinhard Jahn, Ph.D., and Assistant Investigators Sankar Ghosh, Ph.D., and Tsutomu Tanabe, Ph.D., at Yale University; Assistant Investigator Geoffrey M. Duyk, M.D., Ph.D., at Harvard Medical School; Associate Investigator Nancy L. Craig, Ph.D., at the Johns Hopkins University; Assistant Investigator Matthew L. Thomas, Ph.D., at Washington University; Investigator Marc G. Caron, Ph.D., and Assistant Investigator Laura I. Davis, Ph.D., at Duke University; Assistant Investigator Michael H. Malim, Ph.D., at the University of Pennsylvania; and Associate Investigator Masashi Yanagisawa, M.D., Ph.D., at the University of Texas Southwestern Medical Center at Dallas.

INVESTIGATORS

The following is a list of the investigators who were either appointed to or promoted within (marked by an asterisk) the Institute's research staff:

Investigators

Marc G. Caron, Ph.D., *Duke University*

Peter Cresswell, Ph.D., *Yale University School of Medicine*

*Mark M. Davis, Ph.D., *Stanford University School of Medicine*

Pietro De Camilli, M.D., *Yale University School of Medicine*

*Nathaniel Heintz, Ph.D., *The Rockefeller University*

*Louis M. Kunkel, Ph.D., *The Children's Hospital, Boston*

*Stephen A. Liebhaber, M.D., *University of Pennsylvania School of Medicine*

*Roger M. Perlmutter, M.D., Ph.D., *University of Washington, Seattle*

Larry Simpson, Ph.D., *University of California, Los Angeles*

*Thomas C. Südhof, M.D., *University of Texas Southwestern Medical Center at Dallas*

Associate Investigators

*Bruce A. Beutler, M.D., *University of Texas Southwestern Medical Center at Dallas*

Michael P. Bevilacqua, M.D., Ph.D., *University of California, San Diego*

Nancy L. Craig, Ph.D., *The Johns Hopkins University School of Medicine*

*Claude Desplan, Ph.D., *The Rockefeller University*

Scott D. Emr, Ph.D., *University of California, San Diego*

Reinhard Jahn, Ph.D., *Yale University School of Medicine*

*Dan R. Littman, M.D., Ph.D., *University of California, San Francisco*

*Jeremy Nathans, M.D., Ph.D., *The Johns Hopkins University School of Medicine*

*B. Matija Peterlin, M.D., *University of California, San Francisco*

*Stephen T. Reeders, M.D., *Yale University School of Medicine*

*Paul W. Sternberg, Ph.D., *California Institute of Technology*

Masashi Yanagisawa, M.D., Ph.D., *University of Texas Southwestern Medical Center at Dallas*

Assistant Investigators

Laura I. Davis, Ph.D., *Duke University*

Geoffrey M. Duyk, M.D., Ph.D., *Harvard Medical School*

Sankar Ghosh, Ph.D., *Yale University School of Medicine*

Michael H. Malim, Ph.D., *University of Pennsylvania School of Medicine*

Tsutomu Tanabe, Ph.D., *Yale University School of Medicine*

Matthew L. Thomas, Ph.D., *Washington University, St. Louis*

AWARDS AND RECOGNITIONS

The research accomplishments of the Institute's investigators have been recognized in many ways—through prestigious awards, election to distinguished professional societies, and invitations to present prestigious named lectures around the world. Only a brief selection from among these honors is included below. Certain aspects of their research have also received support through such distinguished funding mechanisms as the Pew Charitable Trusts, Searle Scholars, and the Javits, MERIT, and Outstanding Investigator Grant awards of the National Institutes of Health, some of which extend into the next century.

Investigators Steven L. McKnight, Randy W. Schekman, Paul B. Sigler, and Raymond L. White were elected to the National Academy of Sciences. Investigator Stuart H. Orkin was elected to the Institute of Medicine.

Investigator Yuet Wai Kan was a recipient of the 1991 Albert Lasker Clinical Medical Research Award and the Cotlove Award of the Academy of Clinical Laboratory Physicians and Scientists. He, as well as Investigators Philippa Marrack and Joan A. Steitz, received the Christopher Columbus Discovery Award for Biomedical Research. Dr. Steitz also received the Golden Plate Award of the American Academy of Achievement.

The 1992 Rank Prize for Opto-electronics was shared by Investigator J. Anthony Movshon with Drs. Edward H. Adelson, William T. Newsome III, and Semir M. Zeki. Investigator Mario R. Capecchi received the Fifth Annual Bristol-Myers Squibb Award for Distinguished Achievement in Neuroscience Research with Drs. Sydney Brenner and Seymour Benzer. The 1992 Pfizer Award in Enzyme Chemistry was won by Investigator Carl O. Pabo.

Associate Investigator Richard L. Huganir received the 1991 Young Investigator Award of the Society for Neuroscience. The 1992 Young Investigator Award of the Biophysical Society was given to Associate Investigator Richard W. Aldrich.

Investigator David L. Garbers was awarded the Amory Prize of the American Academy of the Arts and Sciences. Investigator Francis S. Collins received the 1991 National Medical Research Award of the National Health Council. Among his other awards during the year were the E. Mead Johnson Award for Research in Pediatrics, the third Ray and Robert Kroc Award for Excellence in Biomedical Research, and, from his institution, the Distinguished Faculty Achievement Award of the University of Michigan. Investigator Raymond L. White also was a recipient of the 1991 National Medical Research Award of the National Health Council and received

the 1992 Lewis S. Rosenstiel Award at Brandeis University for Distinguished Work in Basic Medical Sciences.

Investigator Bernardo Nadal-Ginard received the Basic Research Prize of the American Heart Association. The Arthritis Foundation awarded Investigator John P. Atkinson the Lee C. Howley, Sr., prize for arthritis research. Dr. Atkinson's skills as an educator were acknowledged by the Distinguished Service Teaching Award of the Washington University School of Medicine. Investigator Ronald M. Evans received the Osborne and Mendel Award of the American Institute of Nutrition and also gave the Rita Levi Montalcini Lecture of the Fidia Research Foundation. The International Congress on Inflammation presented the ALM Young Investigator Award to Associate Investigator Michael P. Bevilacqua. The Emilio Trabucchi Foundation Medal was awarded to Investigator Kevin P. Campbell for extraordinary achievements in the field of Ca^{2+} channels. Investigator C. Thomas Caskey received both the Service Merchandise Leadership Award of the Muscular Dystrophy Association and the Wadsworth Award of the New York State Department of Health.

Investigator Johann Deisenhofer was awarded the Bavarian Order of Merit. The City of Medicine Award was presented in Durham, North Carolina, to Senior Investigator Philip Leder, Investigator Robert J. Lefkowitz, and Scientific Review Board member Alfred G. Gilman. Dr. Leder also received the Ernst W. Bertner Award of the M.D. Anderson Cancer Center. Dr. Lefkowitz was also honored with the Bristol-Myers Squibb Award for Distinguished Achievement in Cardiovascular Research, the Giovanni Lorenzini Prize for Basic Biomedical Research, and the Alumni Award for Distinguished Achievement in Medicine by Columbia University College of Physicians and Surgeons.

The Warner-Lambert/Parke-Davis Award in Experimental Pathology was given to Assistant Investigator John B. Lowe. Investigator Louis M. Kunkel received the 1991 Sanremo International Award for Genetic Research. Investigator Jonathan G. Seidman shared the 1991 Fifth Annual Pasarow Foundation Award for Research in Cardiovascular Medical Sciences with Dr. Christine Seidman. Investigator Michael J. Welsh received the Doris F. Tulcin Cystic Fibrosis Research Award. Senior Investigator Eric R. Kandel was honored by the Jean-Louis Signoret Prize on Memory of the Fondation Ipsen, Paris; by the John P. McGovern Award in Behavioral Neuroscience; and by the Warren Triennial Prize of the Massachusetts General Hospital.

Investigator King-Wai Yau received the Friedenwald Award of the Association for Research in Vision and Ophthalmology. Investigator Roger Y. Tsien received the W. Alden Spencer Award from Columbia University for research in neurology and behavior. The Association for Research in Vision and Ophthalmology presented Associate Investigator Jeremy Nathans with the Cogan Award. He also received the Alcon Research Institute Award for Vision Research. The Robert J. and Claire Pasarow Foundation Award was given to Investigator Harold M. Weintraub. Assistant Investigator David G. Schatz received the Cheryl Whitlock Prize for his outstanding contribution to the fields of hematopoiesis and leukemogenesis by an individual during training.

Investigator Richard Axel received the Ninth Annual Sense of Smell Award of the Fragrance Research Fund; the Science Pour L'Art Award from LVMH (Moet Hennessy-Louis Vuitton, Corp.), together with Dr. Linda Buck; and the John Jay Award for Distinguished Professional Achievement, Alma Mater, from Columbia University. Columbia University also presented the Stevens Triennial Prize of the College of Physicians and Surgeons to Investigator Wayne A. Hendrickson. The University of Calgary recognized Investigator Graeme I. Bell with its Outstanding Alumni Award. Associate Investigator Gary J. Nabel received an official citation from the State of Connecticut General Assembly for contributions to human gene therapy.

Honorary doctoral degrees were given to Investigator Francis S. Collins by Yale University; to Investigator Charles A. Janeway, Jr., by the Copernicus School of Medicine in Cracow, Poland; to Investigator Bernardo Nadal-Ginard by the Universitat de les Illes Balears in Palma de Malloica, Spain; to Investigator Joan A. Steitz by Trinity College and by Harvard University; and to Investigator Irving L. Weissman by Montana State University.

The American Society for Clinical Investigation elected Associate Investigators Michael R. Lerner, Gary J. Nabel, and Rebecca A. Taub to membership. The American Academy of Arts and Sciences elected Investigators Max D. Cooper, David L. Garbers, Wayne A. Hendrickson, Thomas M. Jessell, Philippa Marrack, Steven L. McKnight, Gerald M. Rubin, and Paul B. Sigler as fellows. Investigators Francis S. Collins and Stephen A. Liebhaber and Associate Investigator Craig B. Thompson were elected to the American Association of Physicians. Investigator Joan A. Steitz became a member of the American Philosophical Society. Investigator Thomas R. Cech is now Associate Member of the European Molecular Biology Organization. Associate Investigator Stephen T. Warren is a member of the Human Genome Organization. Investigator Günter Blobel was elected to Academia Europaea and Investigator Michael M.-C.

Lai to the Academia Sinica (Taiwan). Investigators C. Thomas Caskey and Max D. Cooper were elected Fellows of the Royal Society of Medicine Foundation, Inc.

The Institute's Investigators have continued to participate actively in their professional societies and to contribute their efforts to the larger scientific community. For example, Associate Investigator Arthur Weiss served as President of the Western Region of the American College of Physicians; Investigator Charles A. Janeway, Jr., is a councillor of the American Association of Immunologists and a member of the Board of Directors of FASEB; Associate Investigator Donald E. Ganem serves as a councillor of the American Society for Virology; and Associate Investigator Bruce A. Beutler was President of the International Congress on Tumor Necrosis Factor and Related Cytokines. HHMI investigators serve on advisory boards and reviewing panels for such agencies and organizations as the National Institutes of Health, the American Heart Association, the American Red Cross, the Searle Scholars and the Pew Scholars Programs, Jane Coffin Childs Memorial Fund, David and Lucile Packard Foundation, and the Damon Runyon and Walter Winchell Cancer Research Fund. Senior Investigator Donald F. Steiner served as Chairman of the Board of Scientific Counselors of the National Institute of Diabetes and Digestive and Kidney Diseases, NIH; Investigators Uta Francke and Charles A. Janeway, Jr., served as chairpersons of the Mammalian Genetics and the Immunobiology Study Sections of NIH, respectively.

INTERNATIONAL RESEARCH SCHOLARS

Through grants to their respective institutions, the Institute supported research projects in the laboratories of 10 scientists at 4 institutions in Mexico and 14 at 7 institutions in Canada.

Mexico

Carlos F. Arias, Ph.D., *Biotechnology Institute, National Autonomous University of Mexico, Cuernavaca*

Edmundo Calva, Ph.D., *Biotechnology Institute, National Autonomous University of Mexico, Cuernavaca*

Gabriel Cota, Ph.D., *National Polytechnic Institute, Mexico City*

Alberto Darszon, Ph.D., *Biotechnology Institute, National Autonomous University of Mexico, Cuernavaca*

Gabriel Guarneros-Peña, Ph.D., *National Polytechnic Institute, Mexico City*

Luis R. Herrera-Estrella, Ph.D., *National Polytechnic Institute, Irapuato*

Paul M. Lizardi, Ph.D., *Biotechnology Institute, National Autonomous University of Mexico, Cuernavaca*

Esther Orozco, Ph.D., *National Polytechnic Institute, Mexico City*

Lourival D. Possani, Ph.D., *Biotechnology Institute, National Autonomous University of Mexico, Cuernavaca*

Ranulfo Romo, M.D., Ph.D., *Institute of Cellular Physiology, National Autonomous University of Mexico, Mexico City*

Canada

Alan Bernstein, Ph.D., *Samuel Lunenfeld Research Institute of Mount Sinai Hospital, Toronto, and the University of Toronto*

B. Brett Finlay, Ph.D., *University of British Columbia, Vancouver*

Jack F. Greenblatt, Ph.D., *University of Toronto*

Sergio Grinstein, Ph.D., *The Research Institute of the Hospital for Sick Children, Toronto, and the University of Toronto*

Philippe Gros, Ph.D., *McGill University, Montreal*

Alexandra L. Joyner, Ph.D., *Samuel Lunenfeld Research Institute of Mount Sinai Hospital, Toronto, and the University of Toronto*

James D. McGhee, Ph.D., *University of Calgary, Alberta*

Tim Mosmann, Ph.D., *University of Alberta, Edmonton*

Tony Pawson, Ph.D., *Samuel Lunenfeld Research Institute of Mount Sinai Hospital, Toronto, and the University of Toronto*

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Lap-Chee Tsui, Ph.D., *The Research Institute of the Hospital for Sick Children, Toronto, and the University of Toronto*

W. Maxwell Cowan, M.D., Ph.D.
Vice President and Chief Scientific Officer

HOWARD HUGHES MEDICAL INSTITUTE
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National Institutes of Health

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University of Alberta

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Abby Rockefeller Mauzé Chair in Experimental
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Member, Cellular Biochemistry and Biophysics
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Memorial Sloan-Kettering Cancer Center

Michael G. Rossmann, Ph.D.
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HOWARD HUGHES MEDICAL INSTITUTE
SENIOR INVESTIGATORS, INVESTIGATORS, ASSOCIATE INVESTIGATORS, AND ASSISTANT INVESTIGATORS
SEPTEMBER 1, 1991–AUGUST 31, 1992

SENIOR INVESTIGATORS
Kandel, Eric R., M.D.
Leder, Philip, M.D.
Nathans, Daniel, M.D.
Steiner, Donald F., M.D.

INVESTIGATORS
Adams, Paul R., Ph.D.
Alt, Frederick W., Ph.D.
Artavanis-Tsakonas, Spyridon, Ph.D.
Atkinson, John P., M.D.
Axel, Richard, M.D.
Beach, David H., Ph.D.
Beaudet, Arthur L., M.D.
Bell, Graeme I., Ph.D.
Bennett, G. Vann, M.D., Ph.D.
Bevan, Michael J., Ph.D.
Blackshear, Perry J., M.D., D.Phil.
Blobel, Günter, M.D., Ph.D.
Bloom, Barry R., Ph.D.
Brugge, Joan S., Ph.D.
Campbell, Kevin P., Ph.D.
Capecchi, Mario R., Ph.D.
Caron, Marc G., Ph.D.
Caskey, C. Thomas, M.D.
Cech, Thomas R., Ph.D.
Chin, William W., M.D.
Collins, Francis S., M.D., Ph.D.
Cooper, Max D., M.D.
Cresswell, Peter, Ph.D.
Davis, Mark M., Ph.D.
De Camilli, Pietro, M.D.
Deisenhofer, Johann, Ph.D.
Donelson, John E., Ph.D.
Dreyfuss, Gideon, Ph.D.
Esmon, Charles T., Ph.D.
Evans, Ronald M., Ph.D.
Exton, John H., M.D., Ph.D.
Fischer Lindahl, Kirsten, Ph.D.
Flavell, Richard A., Ph.D.
Francke, Uta, M.D.
Fuchs, Elaine, Ph.D.
Garbers, David L., Ph.D.
Gesteland, Raymond F., Ph.D.
Gething, Mary-Jane H., Ph.D.
Glomset, John A., M.D.
Goodman, Corey S., Ph.D.
Greene, Warner C., M.D., Ph.D.
Habener, Joel F., M.D.

Harrison, Stephen C., Ph.D.
Heintz, Nathaniel, Ph.D.
Hendrickson, Wayne A., Ph.D.
Henikoff, Steven, Ph.D.
Horvitz, H. Robert, Ph.D.
Hynes, Richard O., Ph.D.
Jan, Lily Y., Ph.D.
Jan, Yuh Nung, Ph.D.
Janeway, Charles A., Jr., M.D.
Jessell, Thomas M., Ph.D.
Kaback, H. Ronald, M.D.
Kan, Yuet Wai, M.D.
Kappler, John W., Ph.D.
Kaufman, Thomas C., Ph.D.
Kunkel, Louis M., Ph.D.
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Lefkowitz, Robert J., M.D.
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Lindquist, Susan L., Ph.D.
Maller, James L., Ph.D.
Marrack, Philippa, Ph.D.
Massagué, Joan, Ph.D.
Matthews, Brian W., Ph.D., D.Sc.
McKnight, Steven L., Ph.D.
Miller, Christopher, Ph.D.
Movshon, J. Anthony, Ph.D.
Nadal-Ginard, Bernardo, M.D., Ph.D.
Nevins, Joseph R., Ph.D.
Olson, Maynard V., Ph.D.
Orkin, Stuart H., M.D.
Pabo, Carl O., Ph.D.
Palmiter, Richard D., Ph.D.
Perlmutter, Roger M., M.D., Ph.D.
Quiocho, Florante A., Ph.D.
Reichardt, Louis F., Ph.D.
Rosbash, Michael, Ph.D.
Rosenfeld, Michael G., M.D.
Rubin, Gerald M., Ph.D.
Schekman, Randy W., Ph.D.
Schwartz, James H., M.D., Ph.D.
Sedat, John W., Ph.D.
Seidman, Jonathan G., Ph.D.
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Shenk, Thomas E., Ph.D.
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Sigler, Paul B., M.D., Ph.D.
Simpson, Larry, Ph.D.
Spradling, Allan C., Ph.D.
Steitz, Joan A., Ph.D.
Steitz, Thomas A., Ph.D.
Stevens, Charles F., M.D., Ph.D.
Südhof, Thomas C., M.D.
Tilghman, Shirley M., Ph.D.
Tjian, Robert, Ph.D.
Tonegawa, Susumu, Ph.D.
Tsien, Roger Y., Ph.D.
Valle, David L., M.D.
Watterson, D. Martin, Ph.D.
Weintraub, Harold M., M.D., Ph.D.
Weissman, Irving L., M.D.
Welsh, Michael J., M.D.
White, Raymond L., Ph.D.
Wiley, Don C., Ph.D.
Williams, Lewis T., M.D., Ph.D.
Witte, Owen N., M.D.
Woo, Savio L. C., Ph.D.
Yau, King-Wai, Ph.D.
Young, Michael W., Ph.D.
Ziff, Edward B., Ph.D.

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Agard, David A., Ph.D.
Aldrich, Richard W., Ph.D.
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Bevilacqua, Michael P., M.D., Ph.D.
Bottomly, H. Kim, Ph.D.
Chaplin, David D., M.D., Ph.D.
Corden, Jeffry L., Ph.D.
Corey, David P., Ph.D.
Crabtree, Gerald R., M.D.
Craig, Nancy L., Ph.D.
Cullen, Bryan R., Ph.D.
Desiderio, Stephen V., M.D., Ph.D.
Desplan, Claude, Ph.D.
Emr, Scott D., Ph.D.
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Sakonju, Shigeru, Ph.D.
Schatz, David G., Ph.D.
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Thummel, Carl S., Ph.D.
Williams, David A., M.D.
Wilson, James M., M.D., Ph.D.
Wolin, Sandra L., M.D., Ph.D.
Yellen, Gary, Ph.D.

LOCATIONS OF HOWARD HUGHES MEDICAL INSTITUTE LABORATORIES

Alabama	University of Alabama at Birmingham and associated hospitals
California	California Institute of Technology and associated hospitals, Pasadena The Salk Institute for Biological Studies, San Diego Stanford University and the Stanford University Hospital, Palo Alto University of California, Berkeley, and associated hospitals University of California, Los Angeles, and associated hospitals University of California, San Diego, and the UCSD Medical Center University of California, San Francisco, and associated hospitals University of Southern California, Los Angeles, and associated hospitals
Colorado	National Jewish Center for Immunology and Respiratory Medicine, Denver University of Colorado at Boulder and the University's Health Sciences Center University of Colorado Health Sciences Center, Denver, and associated hospitals
Connecticut	Yale University and associated hospitals, New Haven
Georgia	Emory University School of Medicine, Atlanta, and associated hospitals
Illinois	Northwestern University and associated hospitals, Evanston The University of Chicago and The University of Chicago Hospitals
Indiana	Indiana University, Bloomington, and associated hospitals Indiana University School of Medicine, Indianapolis, and associated hospitals
Iowa	The University of Iowa and associated hospitals, Iowa City
Maryland	The Carnegie Institution of Washington, Baltimore, and The Johns Hopkins Hospital The Johns Hopkins University and Hospital, Baltimore
Massachusetts	Brandeis University, Waltham, and associated hospitals Brigham and Women's Hospital, Boston The Children's Hospital, Boston Harvard College, Arts and Sciences, Cambridge Harvard Medical School, Boston Massachusetts General Hospital, Boston Massachusetts Institute of Technology and associated hospitals, Cambridge Tufts University School of Medicine and associated hospitals, Boston University of Massachusetts, Worcester, and associated hospitals
Michigan	University of Michigan and associated hospitals, Ann Arbor
Missouri	Washington University and associated hospitals, St. Louis
New Jersey	Princeton University and associated medical centers, Princeton
New York	Albert Einstein College of Medicine of Yeshiva University, Bronx, and associated hospitals Cold Spring Harbor Laboratory and associated hospitals, Cold Spring Harbor Columbia University and associated hospitals, New York City Cornell University Medical College, New York City Memorial Sloan-Kettering Cancer Center, New York City New York University (Medical Center and Washington Square) and associated hospitals, New York City The Rockefeller University and Rockefeller University Hospital, New York City State University of New York at Stony Brook and University Hospital at Stony Brook
North Carolina	Duke University, including Duke University Medical Center, Durham

Oklahoma	Oklahoma Medical Research Foundation and associated hospitals, Oklahoma City
Oregon	University of Oregon and associated hospitals, Eugene
Pennsylvania	University of Pennsylvania and associated hospitals, Philadelphia
Tennessee	St. Jude Children's Research Hospital, Memphis Vanderbilt University, including Vanderbilt University Hospital, Nashville
Texas	Baylor College of Medicine and associated hospitals, Houston Rice University and associated hospitals, Houston University of Texas Southwestern Medical Center at Dallas and associated hospitals
Utah	University of Utah, including University of Utah Medical Center, Salt Lake City
Washington	Fred Hutchinson Cancer Research Center, Seattle University of Washington and associated hospitals, Seattle
Wisconsin	University of Wisconsin–Madison, and associated hospitals

OTHER INSTITUTE FACILITIES

Maryland	HHMI-NIH Cloister at the Mary Woodard Lasker Center for Science and Education on the NIH campus, Bethesda
New York	Synchrotron Beam Lines, Brookhaven National Laboratory (under construction)

HOWARD HUGHES MEDICAL INSTITUTE

SCIENTIFIC MEETINGS

September 22–25, 1991   Signal Transduction and Its Consequences

October 13–16, 1991   Transcriptional, Translational, and Post-translational Regulation

February 23–26, 1992   The Analysis of Structure

March 8–11, 1992   Molecular Approaches to Development

March 29–April 1, 1992   Receptors and the Signals They Transduce

May 17–20, 1992   Stem Cells, Lineages, and the Determination of Cellular Phenotype

SCIENTIFIC CONFERENCES

October 4–6, 1991   Molecular Analysis of Chromosome Abnormalities in Human Leukemia and Lymphoma
Dr. Janet D. Rowley, *organizer*

November 17–20, 1991   Segment Polarity Gene Workshop
Drs. Roeland Nusse and Norbert Perrimon, *organizers*

December 8–10, 1991   HHMI Biopolymer Facilities Workshop
Dr. Dan W. Crimmins, *organizer*

CELL BIOLOGY AND REGULATION

The Program in Cell Biology and Regulation is largely concerned with the structure and biology of individual cells, the factors that regulate their normal growth and distinctive functions, and the ways in which cells interact with each other. It is a continuation of the Institute's oldest research program. Formed in 1976 as the Metabolic Regulation Program, it was renamed in 1985 to reflect the Institute's growing research activity in the area of molecular cell biology.

Investigators working in this program are located at the University of California at Berkeley, at Los Angeles, at San Diego, and at San Francisco; the University of Southern California at Los Angeles; Stanford University; the University of Colorado at Boulder; the University of Colorado Health Sciences Center at Denver; Yale University; the University of Chicago; Northwestern University in Evanston; the University of Iowa College of Medicine in Iowa City; the Johns Hopkins University; the Carnegie Institution of Washington at Baltimore; the University of Massachusetts at Worcester; Brigham and Women's Hospital in Boston; Massachusetts General Hospital; Tufts University School of Medicine in Boston; the Massachusetts Institute of Technology; the University of Michigan Medical School; Washington University School of Medicine in St. Louis; Princeton University; Cold Spring Harbor Laboratory; Rockefeller University; Memorial Sloan-Kettering Cancer Center in New York City; Cornell University Medical College in New York City; Duke University School of Medicine; Oklahoma Medical Research Foundation in Oklahoma City; the University of Pennsylvania; St. Jude Children's Research Hospital in Memphis; Vanderbilt University; the University of Texas Southwestern Medical Center at Dallas; Rice University; the University of Washington at Seattle; and the University of Wisconsin–Madison.

Research of Investigator G. Vann Bennett, M.D., Ph.D. (Duke University) and his colleagues has focused on the structure, regulation, and identification of the nearest neighbors of ankyrins, a family of structural proteins strategically located on the cytoplasmic surface of the plasma membrane with recognition sites for both membrane-spanning integral proteins and cytoplasmic structural proteins. A major class of ankyrin-binding proteins has been identified in adult rat brain and determined to be related closely to a membrane-spanning neural cell adhesion molecule in the immunoglobulin superfamily previously implicated in axonal bundling in development of embryonic brain. A physiological consequence of the convergence of cytoskeletal, transmembrane, and intercellular connections in adult brain may be stabilization of the structure of the nervous system so that this intricate arrangement of cells can survive the traumas of everyday life.

Cells in the body are attached to adhesive proteins via the cell surface receptors called integrins. This attachment is vital for proper cell function; plays important roles in embryogenesis, hemostasis, and wound healing; and is aberrant in thrombosis and cancer. The laboratory of Investigator Richard O. Hynes, Ph.D. (Massachusetts Institute of Technology) studies several of the adhesive proteins, especially the fibronectins. In the past year the group has made progress in 1) developing strains of mice with alterations in genes encoding fibronectins and integrins, and another adhesive protein, P-selectin, involved in inflammation; 2) analyzing the roles in embryonic development of integrins and fibronectins in both fruit flies and mice; 3) understanding the regulation of expression and function of these proteins; and 4) analyzing the nature of connections between fibronectins, integrins, and the cytoskeleton. The laboratory continues to study the molecular details of these proteins and their roles in normal physiology and abnormal pathology.

Vascular endothelial cells form a nearly continuous cobblestone-like lining of blood vessels throughout the body. The laboratory of Associate Investigator Michael P. Bevilacqua, M.D., Ph.D. (University of California, San Diego) studies the mechanisms by which leukocytes (white blood cells) bind to the vascular endothelium. Although beneficial in fighting infection and in wound healing, this binding process can contribute to human disease under certain conditions, such as in heart attacks, acute lung injury, and rheumatoid arthritis. One group of molecules that support the binding of leukocytes to the endothelial lining are the selectins. E-selectin is displayed on the surface of endothelium at sites of inflammation and binds to sugars on the surface of leukocytes. Dr. Bevilacqua's laboratory is using synthetic sugars to assess binding to selectins directly and to identify anti-inflammatory therapeutic agents. In a related project, the efforts of the group have demonstrated that blood-borne tumor cells, including those derived from colon cancers and melanomas, may use endothelial adhesion molecules to spread to distant sites (metastasize). Efforts are under way to understand the molecular mechanisms of these processes, in the hope of identifying new anticancer therapies.

Understanding the mechanism of information transfer from one to three dimensions, as in the folding of proteins, remains a major unsolved problem in molecular biology. The laboratory of Assistant Investigator Peter S. Kim, Ph.D. (Massachusetts Institute of Technology) uses small proteins and protein fragments to investigate the pathway of protein folding and the structures of folding intermediates. The interaction between macromolecules is central to much of molecular physiology. The group is particularly interested in the leucine zipper class of DNA-binding proteins, and they wish to understand the code for specificity and stability of leucine zipper interactions. Postulated rules involved in protein folding and macromolecular recognition are being tested by trying to design amino acid sequences that fold into specific conformations and/or that interact in a predetermined manner with other molecules.

Studies in the laboratory of Investigator John A. Glomset, M.D. (University of Washington) have provided evidence regarding the influence of poly-unsaturated fatty acids on the structure of animal cell membranes, the characteristics of a key enzyme in a newly discovered pathway of membrane phospholipid biosynthesis, and the role of the low-density lipoprotein receptor in promoting the delivery of arachidonic acid for eicosanoic production in human blood monocytes. Further experiments are under way in each of these areas, as well as studies designed to explore the association of proteins that contain covalently bound isoprenoid groups with cell membranes.

The most significant achievement of the research of Investigator Kevin P. Campbell, Ph.D. (University of Iowa) and his colleagues during the past year has been the demonstration that dystroglycan (43/156-kDa dystrophin-associated glycoprotein) is a novel laminin-binding glycoprotein and the suggestion that the function of the dystrophin-glycoprotein complex is to provide a linkage between the subsarcolemma cytoskeleton and the extracellular matrix. The findings of this group strongly support the hypothesis that in Duchenne muscular dystrophy a dramatic reduction in dystroglycan leads to the loss of a linkage between the sarcolemma and extracellular matrix, and this may render muscle fibers more susceptible to necrosis or may disrupt the integrity of muscle.

In the past year, three important discoveries have been made in the laboratory of Investigator Günter Blobel, M.D., Ph.D. (Rockefeller University). First, protein-conducting channels have been shown to be gated open by the signal sequence portion of the protein that is to be translocated. Thus signal sequences function as ligands to open cognate protein-conducting channels. Second, bidirectional transport of proteins in and out of the nucleus proceeds on striking curvilinear tracks that connect intranuclear transcription and ribonucleoprotein assembly sites to dedicated nuclear pore complexes. Third, two distinct cytosolic fractions have been identified for signal sequence–mediated protein translocation into the nucleus, one for protein targeting to the nuclear pore complex and the other for translocation through the nuclear pore complex.

Eukaryotic cells are so named because the genetic material (DNA) is separated from the rest of the cell by a membranous barrier, the nuclear envelope, that restricts the flow of biologic information. Newly synthesized RNA must pass through this barrier from the nucleus, where it is transcribed, to the cytoplasm, where it is translated into protein. Conversely, proteins that are destined to function in the nucleus must be imported from their site of synthesis in the cytoplasm. Many of these proteins affect cell growth through transcription, and their entry into the nucleus is regulated in response to extracellular signals. All nucleocytoplasmic transport proceeds through channels called nuclear pore complexes. Proteins within these channels must be able to identify and transport only a specific subset of cellular proteins and RNAs. The work of Assistant Investigator Laura I. Davis, Ph.D. (Duke University) and her colleagues is aimed at understanding how this process is regulated. They work with yeast cells, because although yeast are simple enough to provide a genetic system as powerful as in bacteria, they follow many of the same rules as do higher eukaryotes. Dr. Davis's group has identified several nuclear pore complex proteins. Inactivation of one of these proteins (NUP1) by mutation showed that it is required for RNA export and protein import. They are now using both biochemical and genetic approaches to identify other proteins involved in this process, by virtue of their functional and/or physical interaction with NUP1.

A novel protein kinase encoded by the yeast *VPS15* gene and a phosphatidylinositol 3-kinase encoded by the *VPS34* gene are essential for protein sorting to the lysosome-like vacuole in yeast. Genetic and biochemical characterization of the *VPS15* and *VPS34* gene products has demonstrated that these proteins are components of a membrane-associated hetero-oligomeric protein complex. Studies by Associate Investigator Scott D. Emr, Ph.D. (University of California, San Diego) and his colleagues of the role of this protein kinase and lipid kinase complex in regulating intracellular protein traffic are beginning to provide new and unexpected insights into the mechanisms governing

protein sorting within the eukaryotic secretory pathway.

Protein secretion is a fundamental and evolutionarily conserved process in eukaryotic cells. This process has been studied in baker's yeast by the laboratory of Investigator Randy W. Schekman, Ph.D. (University of California, Berkeley) by taking advantage of facile genetic and molecular cloning techniques together with the prospect of large-scale biochemical characterization. Genes and gene products have been identified that are implicated in the early phases of protein secretion. New membrane proteins are believed to form a complex that allows secretory proteins, which generally are water soluble, to pass through a water-insoluble membrane. The proteins implicated in this process may be extracted from membranes using mild detergents and then reconstituted into artificial membranes that reproduce the translocation process. A large and complex family of genes has been discovered that governs the intracellular movement of proteins contained within membrane-enclosed particles. This phase of the secretory pathway has also been reproduced in a cell-free system and has been shown to rely on the gene products that are required in living cells. The combination of genetic and biochemical approaches offers the prospect that each of the protein molecules involved in the secretory pathway may be isolated in pure form and examined in simplified reactions.

Assistant Investigator James M. Cunningham, M.D. (Brigham and Women's Hospital) and his colleagues have investigated the properties of a transporter of cationic amino acids that serves as a receptor for leukemogenic retroviruses in mice. They have shown that susceptibility to infection and uptake of cationic amino acids is reduced in virus-infected cells as a consequence of intracellular binding of the transporter to newly synthesized envelope. In addition, they have identified two related proteins that are also transporters of cationic amino acids but demonstrate different kinetic properties and tissue expression. They are studying the importance of one of these transporters in supplying arginine to macrophages that produce nitric oxide, a molecule that can mediate communication between cells and enhance the host defense against intracellular pathogens and tumor cells. The production of nitric oxide in cells infected by murine leukemia virus is also under investigation.

The five genes encoding subunits of the DNA polymerase III holoenzyme that were previously identified by Assistant Investigator Michael E. O'Donnell, Ph.D. (Cornell University Medical College) and his colleagues have now been sequenced and cloned into expression vectors. The subunits were overproduced and purified in 100-mg quantities. Biochemical analyses have distinguished individual assays for each subunit, and physical studies have elucidated many of the principal subunit-subunit contacts in the holoenzyme. Sequence comparisons and the limited functional information available in the less-advanced eukaryotic systems suggest that the multiprotein chromosomal polymerase of both yeast and humans is quite similar to the *Escherichia coli* polymerase III holoenzyme. The large amount of protein generated by Dr. O'Donnell's group has made possible a collaboration with Dr. John Kuriyan's HHMI laboratory at Rockefeller University on the x-ray structure of the holoenzyme. The structure of the β clamp was solved, and its ring shape fits nicely with the biochemical prediction of a doughnut appearance. In another project with Epstein-Barr virus, the EBNA1 origin-binding protein was found to loop out the origin DNA, implying that activation of replication may be similar to transcription activation, which involves loops induced by enhancer binding proteins. EBNA1 was also found to distort the DNA helix at the origin, presumably an early step in preparing the DNA for the host replication machinery.

Investigator H. Ronald Kaback, M.D. (University of California, Los Angeles) and his colleagues are studying the lactose permease of *Escherichia coli*, an extensively characterized membrane protein with 12 transmembrane α-helical domains. The permease is a paradigm for a large class of transport proteins that utilize energy to drive the accumulation of sugars, amino acids, and other substances against concentration gradients. Although it is particularly difficult to obtain high-resolution structural information with this class of proteins, recent experiments have begun to provide clues regarding the arrangement and interaction of the helices in lactose permease. Previously the laboratory had constructed a mutant form of the lactose permease in which all of the neutral cysteine residues were replaced by other neutral residues, with very little loss of activity. New variants of this mutant now have been made in which each of the eight charged residues (four positive and four negative) that are thought to be located within the transmembrane helices were replaced by a cysteine residue, and they are all completely inactive. However, when all possible combinations of negatively and positively charged amino acid residues are replaced pairwise with cysteine, two double mutants exhibit significant activity. Although there are important phenomenological differences between the two sets of double mutants, in both instances a negatively and a positively charged residue clearly interact function-

ally, suggesting that these two pairs of residues may form a salt bridge in the three-dimensional structure of the permease. Therefore it is possible that the region of the protein thought to be helix VII, which contains two of these charged residues (aspartic acid), may be in close proximity to the regions thought to be helices X and XI, which contain the other two interacting residues (lysine).

Associate Investigator Linda J. Pike, Ph.D. (Washington University) and her colleagues are interested in the mechanism of action of growth factors. They have shown that the activity of an intracellular enzyme, a phosphatidylinositol kinase, is stimulated in response to epidermal growth factor (EGF). This enzyme has been purified and cloned using recombinant DNA technology. Other studies have shown that prolonged treatment of cells with EGF renders them insensitive to further stimulation by that growth factor, a phenomenon termed desensitization. The cell surface receptor for EGF is a single polypeptide chain. Upon binding of EGF, two polypeptide chains associate to form a receptor dimer and mediate the biological effects of the hormone. In desensitized cells, EGF receptor dimer formation is blocked, explaining the inability of the cells to respond to the growth factor. The protein kinase p34^{cdc2} has been shown to phosphorylate the EGF receptor and may be responsible for inducing receptor desensitization.

The laboratory of Assistant Investigator Roger J. Davis, Ph.D. (University of Massachusetts) is focused on the investigation of the molecular basis of the interaction of cell surface receptors with polypeptide growth factors. In the past year the laboratory has investigated the role of the phosphorylation of a serine residue located within the carboxyl-terminal domain of the EGF receptor. Phosphorylation at this site was found to account for the process of homologous desensitization of the receptor. Mutations at this negative regulatory site blocked desensitization and resulted in the formation of tumors in animals. In other research, a family of human protein-serine/threonine kinases that are regulated by EGF were molecularly cloned and characterized. These protein kinases were shown to translocate into the nucleus of EGF-treated cells and to activate the transcription factor c-Myc. These findings establish a pathway of signal transduction from a cell surface receptor to the regulation of gene expression in the nucleus.

Associate Investigator Nancy L. Craig, Ph.D. (Johns Hopkins University) and her colleagues are dissecting the transposition mechanism of the bacterial transposon Tn7, focusing on defining the macromolecular interactions and the DNA strand cleavage and joining reactions that mediate this reaction. They are using both genetic and biochemical methods to probe their hypothesis that TnsB, a sequence-specific DNA-binding protein that interacts with the Tn7 ends, contains the catalytic sites for recombination. They are looking for "gain-of-function" TnsB mutants that will reveal these activities and are also examining the ability of TnsB and other Tn7 recombination proteins to react with novel DNA substrates that mimic transposition intermediates.

Cystic fibrosis is a common genetic disease caused by mutations in the gene encoding the cystic fibrosis transmembrane conductance regulator (CFTR). Investigator Michael J. Welsh, M.D. (University of Iowa) and his colleagues have shown that CFTR is a chloride channel that is involved in the secretion of chloride ions by the cells affected by cystic fibrosis. Their work has demonstrated that the opening and closing of this channel is controlled in novel ways by second messengers within the cells. The group is also studying the mechanisms that cause defective function of CFTR. The results of these studies should yield new insights into the normal biology of CFTR and contribute to knowledge of how mutations in the gene encoding this protein cause disease.

The goal of the research in the laboratory of Assistant Investigator James M. Wilson, M.D., Ph.D. (University of Michigan) is to enhance an understanding of inherited diseases and to develop new treatments based on gene replacement. One disorder under investigation, familial hypercholesterolemia (FH), is caused by a defect in a liver-specific gene responsible for clearing cholesterol from the blood. These patients have severe elevations in blood cholesterol and suffer from coronary artery disease in childhood. Using a rabbit animal model for FH, Dr. Wilson and his colleagues have developed a new approach to treatment that is based on correcting the genetic defect in the patient's own liver cells, which has led to a clinical trial. The other disease under investigation in this laboratory is cystic fibrosis (CF). This inherited disorder causes pathology primarily in the lung and pancreas. The group is attempting to identify the abnormalities that lead to lung disease. They are also developing approaches to therapy based on inhalation of a recombinant virus that carries a normal functioning CF gene.

The overall aim of the research of Investigator Elaine Fuchs, Ph.D. (University of Chicago) and her colleagues is to understand the molecular mechanisms that underlie growth, differentiation, and development in the human epidermis of the skin. In the past year, this laboratory has 1) identified the genetic bases for two human skin diseases, epider-

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molysis bullosa simplex (EBS) and epidermolytic hyperkeratosis (EH), in which patients have point mutations in genes encoding keratins, the major structural proteins of the epidermis; 2) engineered the animal models for these diseases that were fundamental in elucidating the bases for human EBS and EH; 3) engineered deletions and point mutations in the basal epidermal keratins and examined their behavior, leading to a correlation between the severity of EBS and the severity with which a specific mutation causes perturbations in keratin filament structure; 4) identified sequences and transcription factors necessary for gene expression in keratinocytes *in vitro* and in transgenic mice, leading to the development of an expression vector that can target expression of genes to the epidermis of transgenic mice; and 5) shown that TGF-α (transforming growth factor-α), IL-6 (interleukin-6), and TNF-α (tumor necrosis factor-α) play roles in controlling growth and differentiation in epidermal cultures and in transgenic mice that provide important animal models for investigating the mechanisms underlying the early stages of skin carcinogenesis and psoriasis.

Mechanical forces are generated during several processes essential to life, e.g., in the musculoskeletal system during locomotion, the uterus during parturition, the lung during respiration, and the cardiovascular system during circulation. The laboratory of Assistant Investigator Jeffrey F. Bonadio, M.D. (University of Michigan) has investigated the basis of load bearing in the mammalian skeleton and, in particular, has focused on the role of type I collagen and fibrillin in this process. In the course of these studies this group also identified a rational treatment strategy for skeletal fragility and degeneration in the general population, based partly on the regulation of collagen gene expression. The laboratory plans to test the validity of this strategy in animal model systems. Toward this end, the laboratory has discovered a novel method for transferring therapeutic genes to the cells of skeletal tissues, e.g., tendon, ligament, cartilage, and bone.

The endothelins are a family of small peptide hormones with a variety of potent biological activities. The first member of the family was identified earlier by Associate Investigator Masashi Yanagisawa, M.D., Ph.D. (University of Texas Southwestern Medical Center at Dallas) and his colleagues as a strong blood pressure–raising molecule secreted by the cells lining the inner surface of blood vessels (the endothelium). This year, Dr. Yanagisawa's laboratory has initiated two major projects aimed at further characterization of the physiological role and regulation of the endothelins and their receptors. These projects should lead to production and characterization of mice deficient for genes encoding the molecular components of the system and to molecular identification of a key enzyme in the biosynthesis pathway of the active peptides, the endothelin-converting enzyme.

The studies in the laboratory of Investigator Joel F. Habener, M.D. (Massachusetts General Hospital) are directed toward the cellular mechanisms responsible for the cell-specific expression and metabolic regulation of genes encoding polypeptide hormones. The group hypothesizes that interactions of DNA sequence elements and DNA-binding proteins occur in a combinatorial process to provide unique complexes resulting in the activation of transcription of specific genes in phenotypically distinct cells. The studies currently concern the cell-specific and cAMP-mediated activation of the somatostatin and glucagon genes and the cell-specific expression of the angiotensinogen gene in the liver. The group seeks to isolate and characterize structurally cAMP-responsive DNA-binding phosphoproteins and developmentally cell-specific homeodomain proteins and to dissect the molecular interactions of the proteins with the specific DNA sequence elements and other components of the transcriptional machinery.

The purpose of the research program of Senior Investigator Donald F. Steiner, M.D. (University of Chicago) and his colleagues is to gain a more complete understanding of the genetic and molecular mechanisms that underlie the production and actions of insulin and related hormones of the pancreatic islets of Langerhans. The biological functions of neuroendocrine precursor peptides, as exemplified by proinsulin, proglucagon, pro-IAPP (islet amyloid polypeptide), and/or the insulin receptor precursor, and the cell biological and biochemical mechanisms that lead to their proteolytic processing into active hormones, neuropeptides, or receptor molecules are also being intensively studied. The long-term goal is to develop basic molecular insight into the mechanisms regulating the development, growth, and function of the pancreatic islets and thereby eventually to develop new diagnostic and therapeutic approaches to metabolic disorders such as diabetes mellitus.

Insulin is secreted when a fasting animal is given a glucose load. Insulin activates enzymes that promote energy storage and inhibits enzymes that break down energy stores. Thus the phenotype of the cell is reset so the cell is optimally prepared to carry out energy storage functions. There has long been speculation that coordinate control of the transcription of genes involved in these diverse metabolic processes

is mediated through a common transcription factor or metabolic switch protein. Assistant Investigator Maria C. Alexander-Bridges, M.D., Ph.D. (Massachusetts General Hospital) and her colleagues have cloned a trans-acting factor, IRE-ABP (insulin response element-A–binding protein), that is regulated by insulin and nutritional manipulations that result in hyperinsulinemia. This factor interacts with many genes that are regulated during the adaptive response of the organism to hyperinsulinemia. IRE-ABP may be a multifunctional transcriptional regulator that promotes a metabolic switch in response to glucose and insulin.

Investigator Perry J. Blackshear, M.D., D.Phil. (Duke University) and his colleagues are studying the molecular mechanisms of action of insulin and related polypeptide growth factors. They hope eventually to explain the biochemical steps between binding of these hormones to their cell surface receptors and such subsequent events in the cell as the activation of enzymes or the rapid turning on of specific genes. Characterization of these pathways should increase an understanding of the normal physiological responses to these hormones, as well as of the molecular defects that occur in the insulin resistance characteristic of obesity and common types of diabetes mellitus.

Thyroid hormones are essential for the regulation of general metabolism, growth, and development. Investigator William W. Chin, M.D. (Brigham and Women's Hospital) has been studying the molecular mechanisms by which thyroid hormones regulate gene expression. He has focused on the transcriptional factors (thyroid hormone receptors and nuclear auxiliary factors) in this process and has provided important clues regarding the role of ligand (thyroid hormone) and protein-protein interactions. It is hoped that such information will provide insight into hormone regulation of gene expression, in general, and specifically the mechanism of thyroid hormone action. For example, it may permit an understanding of the mechanisms involved in defective signaling in the syndrome of generalized thyroid hormone resistance.

Steroid hormones play essential roles in salt and water balance, sugar metabolism, and reproduction. Their biosynthesis requires the action of a related group of enzymes, and Assistant Investigator Keith L. Parker, M.D., Ph.D. (Duke University) and his colleagues are studying the factors that regulate the production of these enzymes in the adrenal gland. They have defined regulatory elements and proteins that control the expression of these enzymes. One protein interacts with promoter elements from all of the

steroid hydroxylases, implying a pivotal role in their coordinate expression. This protein is one of the nuclear receptor proteins, a diverse group of structurally related proteins that are involved in the responses to such diverse agents as steroids, thyroid hormone, and vitamin D. The same gene that regulates the steroid hydroxylases also plays an important role in early embryonic development through the production of another protein that has both shared and distinct structural features. Ongoing studies of this key transcriptional regulator should provide important insights into the mechanisms that control steroid hormone biosynthesis and may provide novel concepts about the differentiation of steroidogenic tissues.

Many hormones and growth factors act by stimulating the breakdown of specific membrane lipids. This leads to the generation of the signaling molecules controlling the activities of protein kinases that phosphorylate important cellular proteins. Research in the laboratory of Investigator John H. Exton, M.D., Ph.D. (Vanderbilt University) has identified proteins and unexpected mechanisms that are involved in regulating the breakdown of the lipids. The new proteins are some that transduce signals from cell surface receptors for hormones (G proteins) or that catalyze the breakdown of the lipids (phospholipases). The new mechanisms are those involving unexpected components of the G proteins and actions of protein kinases that do not involve phosphorylation.

Guanylyl cyclase receptors exist within the cytoplasm as well as the membranes of most cells. Investigator David L. Garbers, Ph.D. (University of Texas Southwestern Medical Center at Dallas) and his colleagues have found that one receptor, responsible for acute secretory diarrhea when stimulated by bacterial peptides, responds normally to an endogenous peptide named guanylin. The receptor and guanylin also appear to exist in tissues outside the intestine. A cDNA clone for guanylin has now been obtained and is being used to define its function in the intestine as well as in other tissues.

Adrenergic receptors play an important role in the control of cardiovascular function by the central nervous system. During the past year members of the research group of Assistant Investigator Brian K. Kobilka, M.D. (Stanford University) have learned more about the structure of these receptors, how they are synthesized and inserted into a lipid bilayer, and the intracellular targeting and agonist-mediated redistribution of different subtypes of adrenergic receptors. Progress has also been made in understanding how β_2-receptor synthesis may be regulated by a

small peptide-coding sequence immediately in front of the receptor-coding sequence on β_2-receptor mRNA.

Investigator Robert J. Lefkowitz, M.D. (Duke University) and his colleagues report that their major accomplishments over the past year were 1) discovery of mutations of G protein–coupled receptors that constitutively activate the receptors and a delineation of their properties; and 2) discovery of the role of protein isoprenylation in the translocation and function of G protein–coupled receptor kinases, such as βARK (β-adrenergic receptor kinase) and rhodopsin kinase.

Work in the laboratory of Associate Investigator Roeland Nusse, Ph.D. (Stanford University) is focused on a group of genes that exemplify the link between cancer and the control of normal development. The prototypic member of this group is *Wnt-1*, an oncogene in mouse breast cancer, but normally only expressed in the developing brain of a mouse embryo. This research group wishes to understand the mechanism of action of the *Wnt-1* gene family during embryogenesis and to extrapolate these findings to cancerous growth. One model system under study is the fruit fly *Drosophila*, where the homologue of *Wnt-1* is identical to a developmental gene called *wingless*. Because of the extensive genetic analysis of *Drosophila* embryogenesis, the interactions of *Wnt-1*/wingless with other genes can be conveniently studied.

When adenovirus infects human cells, it replicates to produce progeny virus, and the cell is killed. When rodent cells are infected, the viral replication cycle cannot be completed. Some of the infected cells are not killed and become oncogenically transformed. In fact, some adenoviruses induce tumor formation when inoculated into rodents. The laboratory of Investigator Thomas E. Shenk, Ph.D. (Princeton University) seeks to define mechanisms underlying adenovirus gene regulation in infected human cells and tumor induction in rodents. Studies of the regulation of gene expression have focused on the adenovirus E1A protein, the first viral protein expressed within infected cells and the main adenovirus regulatory protein, activating the synthesis of mRNAs from all remaining viral genes. Work is in progress to elucidate the mechanisms by which the E1A protein interacts with cellular regulatory proteins to control mRNA synthesis. Studies of tumor induction have focused on an adenovirus that can induce mammary tumors in rats. The E1A and E1B viral gene products are well known to play a role in adenovirus oncogenesis. However, the formation of mammary tumors re-quires an additional protein encoded by the adenovirus *E4* gene. Work is in progress to probe the function of this newly identified oncoprotein.

Assistant Investigator Sandra L. Wolin, M.D., Ph.D. (Yale University) and her colleagues are interested in understanding post-transcriptional mechanisms for regulating gene expression in eukaryotic cells. One focus of her group has been the factors that affect ribosome movement along mRNAs during translation. Using a method that allows the detection and mapping of paused ribosomes on the mRNA, they have identified a new intermediate in translation initiation in which a fully assembled ribosome pauses over the initiation codon. They are determining what features of mRNA sequence and structure result in ribosome pausing and in frameshifting during the translation of retroviral mRNAs. In a separate project, the laboratory is investigating the structure and function of a conserved class of small cytoplasmic ribonucleoprotein particles, the Ro RNPs, often the targets of autoantibodies in patients with certain rheumatological disorders.

Messenger RNAs (mRNAs), the functional translatable intermediates of gene expression, are formed in the nuclei of eukaryotic cells by extensive and tightly regulated post-transcriptional processing of heterogeneous nuclear RNAs (hnRNAs), the primary RNA polymerase II transcripts. Throughout the time they are in the nucleus, hnRNAs are associated with proteins—the hnRNPs. These proteins influence the structure of hnRNAs and therefore their fate and processing into mRNAs. The hnRNPs are as abundant in growing vertebrate cells as histones, and hnRNA-hnRNP complexes are thus also of interest, because they are major nuclear structures. In the cytoplasm, mRNAs are associated with mRNPs, and these are likely to be involved in the regulation of the translation and stability of mRNAs and in their cellular localization. The goal of Investigator Gideon Dreyfuss, Ph.D. (University of Pennsylvania) and his colleagues is to understand, in molecular detail and cellular architecture, how the post-transcriptional portion of the pathway of gene expression operates in the cell. To do so they investigate the structure, function, and localization of the hnRNP and mRNPs, and hnRNP complexes.

Investigations of experimental models of human disease in the laboratory of Investigator Mary-Jane H. Gething, Ph.D. (University of Texas Southwestern Medical Center at Dallas) grow out of several years of basic research on the biochemical and structural properties of cellular and viral proteins. Experiments involve three systems: 1) human tissue-type plasminogen activator, a serine protease involved in

fibrinolysis, tissue remodeling, and metastasis; 2) the hemagglutinin of influenza virus, which is being used to develop models of autoimmune disease in transgenic mice; and 3) the tumor-suppressor protein p53 and its interaction with cytosolic stress-70 proteins. Basic studies on the cellular role and regulation of protein chaperones are also under study.

The research group headed by Investigator Lewis T. Williams, M.D., Ph.D. (University of California, San Francisco) has shown a connection between two molecules, phosphatidylinositol 3-kinase and p21 ras, that are known to regulate cell growth. They were surprised to find that a set of reactions previously thought to be important for the action of several growth factors are not required for at least one of these factors to stimulate cell proliferation. They also found that a regulatory molecule, Raf-1, plays an essential role in vertebrate embryonic development. Finally, they cloned the gene for a new receptor that is important for the growth of blood vessels.

Work in the laboratory of Associate Investigator Gerald R. Crabtree, M.D. (Stanford University) is directed at understanding how cells achieve their final identity. Beginning with the same genetic information, cellular decisions are made whereby some become blood cells, others brain cells, and so forth. However, the basis of these decisions is only recently becoming understood. One specific area of work in the laboratory involves the T lymphocytes of the immune system that coordinate the activity of other cells to mount an immune response to an invading organism or transplanted tissue. The drug most commonly used to suppress the rejection of a transplant, cyclosporin A, works by interfering with the ability of a T cell to differentiate into an immunologically functional cell. Work in the Crabtree laboratory and in Dr. Stuart Schreiber's laboratory in Boston has led to a precise understanding of how these drugs function and has opened new avenues for the development of more-specific immunosuppressive agents.

The focus of the research of Assistant Investigator Rudolf Grosschedl, Ph.D. (University of California, San Francisco) and his colleagues is the developmental control of gene expression. In particular, gene transfer of wild-type and mutated immunoglobulin μ heavy-chain genes into the mouse germline has indicated that negative regulation of enhancer function is important for tissue-specific gene expression. Moreover, the μ enhancer is capable of altering the accessibility of nuclear factor–binding sites in native chromatin, independent of transcription. With the goal of identifying and characterizing cell lineage-specific regulators of transcription, the

group isolated cDNA clones encoding two novel lymphocyte-specific factors. Lymphoid enhancer-binding factor 1 (LEF-1) is a member of the family of the high-mobility group (HMG) of proteins and participates in the regulation of the T cell receptor α gene enhancer. In binding to DNA, LEF-1 recognizes its target site through contacts in the minor groove in the DNA helix and induces a very sharp bend. Another lineage-specific protein, early B cell factor (EBF), binds a functionally important site in the promoter of the mb-1 gene, which is expressed specifically at early stages of B cell differentiation. EBF was purified, and cDNA clones encoding this protein were isolated. The roles of LEF-1 and EBF for lineage-specific gene expression and cell differentiation are under study.

In the laboratory of Investigator Nathaniel Heintz, Ph.D. (Rockefeller University), high-resolution genetic mapping studies have resulted in the identification of molecular markers in close proximity to genes involved in a specific developmental defect of the mammalian central nervous system and in two neurodegenerative diseases of mice. Efforts to identify a series of tissue-specific, developmentally regulated genes expressed in the mammalian cerebellum have resulted in the cloning of a variety of genes whose expression marks important biological transitions in the mammalian central nervous system and should provide insight into important molecular events controlling development of the mammalian brain. Studies of transcription factor phosphorylation during the cell cycle have been extended to establish that similar mechanisms regulate distinct histone gene transcription factors (Oct-1, H1TF2). These results imply that a common program of posttranslational modifications may regulate S-phase–specific transcription and, perhaps, DNA replication, during the mammalian cell cycle.

Assistant Investigator Richard L. Maas, M.D., Ph.D. (Brigham and Women's Hospital) and his colleagues are investigating the role that certain genes play in the embryonic development of the mammalian eye and kidney. The genes of primary interest contain either of two DNA-binding functions, a paired box or a homeobox, and are therefore likely to be involved in regulating the activity of other genes that actually carry out the steps involved in forming various organs. The PAX6 gene has been identified as being very important in the formation of the human eye. Mutations in it are responsible for aniridia, a disorder of eye development frequently culminating in blindness. Studies are under way to determine how PAX6 functions. In addition, certain steps of kidney development are under study, and the gene

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Hox-1.8, which is expressed early in kidney development and may thus be important in this process, has been found.

The laboratory of Assistant Investigator Ruth Lehmann, Ph.D. (Massachusetts Institute of Technology) explores development in the fruit fly. Essential clues for the establishment of the *Drosophila* body plan are provided to the egg cell during its maturation in the mother fly, and these signals are under study. Some of the maternal gene products are localized to specific egg regions. At the posterior pole, signals required for the formation of the embryonic abdomen and for the determination of germ cells are stored in the form of RNA. Mislocalization of these RNA molecules to new positions within the egg cell can be achieved by exchange of RNA localization signals. Such experiments show that mislocalization of *nanos* RNA is sufficient to induce a second abdomen in the fly and that mislocalization of *oskar* RNA induces germ cell formation.

Research of Assistant Investigator Sean B. Carroll, Ph.D. (University of Wisconsin) and his colleagues is also focused on groups of genes that govern the formation of body structures in *Drosophila* and in other insects as well, with the goal of understanding how body patterns are created and how they evolve. In the past year they have identified several components of a genetic hierarchy that guides the formation of the entire *Drosophila* wing. The *wingless* protein, one member of a gene family involved in the large-scale patterning of various invertebrate and vertebrate structures, appears to be the earliest activity product that regulates the spatial expression of the dorsal-specific *apterous* gene and the wing-specific *vestigial* and *scalloped* genes. In a separate comparative study of many fly species, this laboratory has determined that the molecular regulatory mechanisms guiding pattern formation in these animals are highly conserved and functionally interchangeable, even after 40–80 million years of evolutionary divergence.

The laboratory of Investigator Allan C. Spradling, Ph.D. (Carnegie Institution) examines the role played by changes in genome structure during the development of the fruit fly. Heterochromatic chromosome regions that contain one-quarter of the *Drosophila* DNA sequences are partially lost during the normal development of many somatic cells, including the ovarian nurse cells. It is not known if these dramatic alterations are functionally important. This group is studying the relationship of genomic alterations to the functioning and regulation of stem cells and nurse cells during oogenesis. A largely heterochromatic minichromosome and several genes that disrupt these aspects of oogenesis provide the necessary tools.

Investigator David H. Beach, Ph.D. (Cold Spring Harbor Laboratory) and his colleagues focus on the regulation of the cell division cycle in model organisms such as yeast, but also in mammalian cells. The division cycle of all eukaryotic cells is controlled by a mitotic oscillator that consists of a family of closely related protein kinases. Each kinase has a catalytic subunit and a cyclin regulatory subunit. The group is investigating the cyclin kinases and attempting to understand how their function is integrated into the signal transduction pathway that regulates mitogenesis.

Cells respond to growth regulatory factors only during the G_1 phase of their division cycle, when they prepare to duplicate their chromosomes. However, once DNA replication begins, growth factors are no longer necessary to ensure subsequent steps in cell division. Colony-stimulating factor 1 (CSF-1) induces cell proliferation by binding to specific cell surface receptors which, in turn, generate intracellular signals that govern gene expression. These signals are under study by the laboratory of Investigator Charles J. Sherr, M.D., Ph.D. (St. Jude Children's Research Hospital). Targets of the signaling pathways have been found to include novel G_1 cyclin genes that may control the cell's commitment to DNA synthesis. Genetic changes that perturb either CSF-1–induced signal transduction or cyclin gene expression can contribute to the development of cancer.

In a cell cycle, DNA is first faithfully replicated (S phase) and then distributed to daughter cells at mitosis (M phase). Cells have an elaborate checkpoint before mitosis (in G_2 phase) to make sure all DNA is fully replicated before initiating mitosis by activation of cdc2 kinase, and this is under study by Investigator James L. Maller, Ph.D. (University of Colorado) and his colleagues. The cdc25 phosphatase activates cdc2 only after phosphorylation of the phosphatase itself. Increased phosphorylation is in part a consequence of decreased phosphatase activity against cdc25 itself. A specialized form of cdc2 known as cdk2 is required for metaphase arrest at meiosis II in the unfertilized egg and appears to cooperate with the c-*mos*^{xe} proto-oncogene kinase to execute this function. Interactions between proto-oncogenes and cell cycle control elements are likely to be important in the aberrant cell cycles of cancer cells.

The proliferation of cells is controlled by a balance of positive and negative signals. The system that conveys growth inhibitory signals is similar in design to that which signals cell growth. Both in-

volve factors that circulate between cells and membrane receptors that are coupled to signaling machinery inside the cell. The signals carried by growth-promoting factors have been extensively studied for the past two decades. The growth inhibitors, however, have come to the attention of biologists only recently. Yet they include some of the most widespread and versatile regulators of cell behavior. Investigator Joan Massagué, Ph.D. (Memorial Sloan-Kettering Cancer Center) and his colleagues have identified the proteins that act as the cell membrane receptors for a well-known inhibitor, transforming growth factor-β (TGF-β). Their study should lead to progress in elucidating the nature of the signals that instruct cells to stop proliferation, and may show ways to constrain the unrestricted growth of cancer cells.

Investigator Steven L. McKnight, Ph.D. (Carnegie Institution) and his colleagues have studied mammalian transcription factors. Cultured 3T3-L1 cells can be converted from mitotically proliferative fibroblasts into terminally differentiated adipocytes in response to a discrete set of adipogenic hormones. The early phase of differentiation is marked by acute cell proliferation, whereas the late phase is marked by growth arrest and morphological specialization. A family of transcription factors related to CCAAT/enhancer-binding protein (C/EBP) have been inferred to regulate the process of adipocyte differentiation. Two C/EBP-related proteins, C/EBPβ and C/EBPδ, are induced during the early proliferative phase of the differentiation program in response to two adipogenic hormones. A third protein, C/EBPα, is expressed late during differentiation when cell growth is arrested and the specialized, adipogenic phenotype is manifested. Ectopic expression of C/EBPα in otherwise proliferative cells causes growth arrest. The sequential activities of the three C/EBP proteins are believed to provide important cues along the differentiation pathway.

Associate Investigator J. Evan Sadler, M.D., Ph.D. (Washington University) and his colleagues investigate the regulation of blood coagulation and, particularly, the role of cells in this process. Cells in contact with the blood actively maintain a balance between stimulating and inhibiting blood coagulation. This balance is disrupted during inflammatory processes that are associated with bleeding or thrombosis, such as bacterial infections, and in certain inherited diseases. This laboratory has studied several endothelial cell hemostatic proteins, including von Willebrand factor and thrombomodulin, as well as the interaction of thrombomodulin with the blood-clotting enzyme thrombin. Mutations in the von

Willebrand factor gene were characterized in patients with von Willebrand disease, the most common human, inherited, bleeding disorder. The regulation and structure-function relationships of thrombomodulin and of thrombin were investigated by mutagenesis and characterization of recombinant proteins.

When blood clots form to block blood vessels (thrombosis), the process results in death to the surrounding tissue unless the clot can be broken up (lysed) rapidly. Investigator Charles T. Esmon, Ph.D. (Oklahoma Medical Research Foundation) and his colleagues developed a model for deep vein thrombosis and implicated inflammation as a major contributor to blood clot formation. This model can be used to test therapeutic approaches and to understand the relationship between thrombosis and inflammation. A key to preventing clot formation is to inhibit the enzyme thrombin. Studies performed in the past year have identified some aspects of how thrombin clot-promoting activity is blocked by thrombomodulin, the protein that interacts with thrombin to activate protein C. These studies may contribute to the design of new clot-preventing drugs.

TNF (tumor necrosis factor) is a protein hormone that is of particular importance in the development of shock and tissue injury during the course of infectious diseases. Associate Investigator Bruce A. Beutler, M.D. (University of Texas Southwestern Medical Center at Dallas) and his colleagues have shown that TNF is also constitutively produced within the thymus and placenta of normal animals. Using transgenic mice bearing markers of TNF synthesis and recombinant inhibitors of TNF activity, they are probing the regulation and function of this protein in order to understand its essential actions.

The laboratory of Associate Investigator Donald E. Ganem, M.D. (University of California, San Francisco) studies how viral pathogens replicate in their host cells and cause disease. Particular attention is paid to the hepatitis B viruses (HBVs), which produce acute and chronic liver injury and predispose infected hosts to the development of liver cancer. Work is focused in two main areas: the molecular mechanisms by which these viruses replicate and the processes by which viral infection leads to cancer. Studies of viral replication have revealed that HBVs, like retroviruses, employ reverse transcription to replicate their DNA, and the details of how this occurs are being examined. This work may allow the identification of new targets for antiviral therapy. An experimental animal model is used to explore the development of liver cancer. Cellular

oncogenes are frequently activated by the nearby integration of viral DNA sequences into the host chromosome in this model system. The implicated cellular oncogenes are being cloned and characterized in an attempt to define the molecular basis of hepatocyte growth control.

The laboratory of Investigator Michael M.-C. Lai, M.D., Ph.D. (University of Southern California) has been studying the molecular biology and the disease-inducing mechanisms of two classes of human and animal infectious agents, coronaviruses and hepatitis delta viruses, both of which contain RNA as their genetic materials. These viruses cause common colds, hepatitis, and a variety of gastrointestinal and respiratory diseases; they also serve as a possible model for multiple sclerosis. This group has examined how these viruses grow and make their RNAs, and how their genes work to cause diseases. A unique mechanism of making viral RNA has been found, as well as a mechanism of genetic exchange called RNA recombination, which is an important tool in virus evolution. Progress also has been made in describing the pathway by which virus infects cells. This new knowledge is applicable to a general understanding of the cause and therapy of viral diseases.

Assistant Investigator Karla A. Kirkegaard, Ph.D. (University of Colorado) and her colleagues have found that the inhibition of poliovirus replication by brefeldin A, an inhibitor of protein secretion, and the inhibition of protein secretion by poliovirus infection both point to the normal protein secretion apparatus as the donor of the membranous vesicles associated with poliovirus replication complexes. A quantitative assay for RNA recombination frequency has shown that RNA recombination continues throughout the course of poliovirus infection, unaffected by these major intracellular rearrangements. In other work they observed that the ability to initiate the replicative cycle of L-A, a double-stranded RNA virus of *Saccharomyces cerevisiae*, with RNA synthesized *in vitro* from cloned cDNA should lead to the development of RNA viral genetics in that organism.

The research in the laboratory of Investigator Robert A. Lamb, Ph.D., Sc.D. (Northwestern University) concerns the molecular structure and mechanism of replication of two enveloped viruses, influenza virus and the paramyxovirus SV5 (simian virus 5). The important diseases in humans and animals caused by influenza virus have serious socioeconomic consequences, because influenza continues to occur in regular epidemics, and an occasional pandemic, and is a leading cause of morbidity and mortality. Among the paramyxoviruses are mumps, measles, canine distemper, Newcastle disease of chickens, and rinderpest of cattle, as well as SV5, the prototype used by Dr. Lamb. Research has focused on the structure, function, and mechanism of intracellular transport of integral membrane proteins of the viral envelope and the replication strategy of the viral RNA genome. In particular, emphasis has been placed on understanding the ion channel activity of the influenza virus M_2 protein, a novel activity for a viral protein and believed to be essential for uncoating the virus in cells. This viral ion channel activity is blocked by the antiviral drug amantadine hydrochloride, and Dr. Lamb's work provides a molecular explanation for the drug's effect.

Many disease-causing microorganisms are able to enter and grow inside human cells. Two such bacteria are being investigated by Assistant Investigator Ralph R. Isberg, Ph.D. (Tufts University) and his colleagues in order to determine how intracellular entry and growth occur. One microorganism that causes gastrointestinal disease, *Yersinia pseudotuberculosis*, was found to enter host cells by binding members of the integrin receptor family present on a wide variety of host cells. Uptake of the organism appears to occur by parasitizing the human cell's normal pathway for internalization of macromolecules. Study of the intracellular growth of *Legionella pneumophila*, the causative agent of Legionnaire's disease, found a small region of the bacterial chromosome that contains a gene permitting the organism to enter into the rough endoplasmic reticulum of host phagocytic cells.

The laboratory of Assistant Investigator Richard H. Gomer, Ph.D. (Rice University) is investigating possible mechanisms of cell differentiation and the ways in which cells sense the number of a given cell type that are in an organism. Such mechanisms may play an important role in growth, wound healing, tissue regeneration, and the control of cell proliferation. Using the simple organism *Dictyostelium*, this group employs molecular biologic techniques to search for genes involved in the determination of which one of its three possible types a *Dictyostelium* cell will become. Related studies concern a protein that the cells secrete and simultaneously sense, allowing them to determine whether they are near many or few other cells.

The laboratory of Associate Investigator Gary K. Schoolnik, M.D. (Stanford University) continues to study the ecology and pathogenesis of bacteria important in human disease. Of special importance have been their studies of enteropathogenic *Esche-*

richia coli (EPEC) and *Vibrio cholerae* (the agent of Asiatic cholera). EPEC, a significant cause of childhood diarrhea, grows as free-living, isolated bacteria in contaminated water, but when ingested it rapidly adopts a colonial mode of growth and forms discrete colonies attached to intestinal mucous membranes. New surface structures form on the bacterial surface when it arrives in the intestinal environment, and these small filaments entwine to bind the bacteria into groups, or colonies. The signals that effect this change are under study. The *V. cholerae* organism is under study to determine how the organism has evolved its ability to live in waters with salinity that varies seasonally. The ancestral cholera toxin genes that permit the persistence of the organism in brackish water between epidemics may have acquired a new role as the principal virulence determinants of the bacteria.

ROLE OF IRE-ABP, A POTENTIAL REGULATOR OF THE SWITCH FROM THE FASTED TO THE REFED STATE

Maria C. Alexander-Bridges, M.D., Ph.D., *Assistant Investigator*

When a fasted animal is fed a high-carbohydrate/low-fat diet, an adaptive response occurs that results in a decrease in glucose production and an increase in glucose utilization and storage. This process is initiated by the hormone insulin. Insulin rapidly increases the activity of rate-limiting enzymes that regulate glucose uptake, glycogen synthesis, and lipogenesis and inhibits the activity of enzymes that increase production of glucose. Over a longer period of time, insulin reinforces these changes in enzymatic activity by altering gene transcription. Thus insulin resets the phenotype of the cell so that the cell is optimally prepared to carry out energy storage functions. The marked alterations in enzymatic activity observed in insulin-deficient diabetes result from atrophy of these pathways.

The work in Dr. Alexander-Bridges' laboratory has focused on defining the distal components in the signaling pathway of insulin action on expression of metabolically active genes. Insulin stimulates transcription of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene in 3T3 adipocytes and H35 hepatoma cells. IRE-A, an insulin response element located between nucleotides −480 to −435 in the upstream region of this gene, specifically interacts with IRP-A, an insulin-induced DNA-binding complex. The ability of adipocyte IRP-A to bind IRE-A DNA correlates with the ability of this element to confer insulin-responsive gene transcription. In transgenic animals, GAPDH–growth hormone fusion genes are regulated by nutritional manipulation, such as fasting and refeeding *in vivo*. These observations show that regulation of GAPDH gene expression is mediated at the level of transcription *in vivo*. The magnitude of the effect on transcription correlates well with the effect of similar manipulations on IRP-A–binding activity in the fat and liver of animals that have been fasted and refed a high-carbohydrate diet and in diabetic animals treated with insulin.

Insulin is known to regulate simultaneously the transcription of genes that control diverse aspects of energy metabolism in lipogenic tissues, e.g., fat and liver. The existence of a common transcription factor that coordinates this adaptive response has been hypothesized for some time.

A protein that binds the GAPDH IRE-A element, IRE-ABP, has been cloned. IRE-ABP binds and activates transcription through the GAPDH IRE-A element in cell lines that do not express IRP-A. Two forms of the factor have been isolated. One enhances, the other inhibits, insulin-sensitive gene transcription through the IRE-A element in insulin-sensitive cell lines.

The high-mobility group (HMG) box domain of the IRE-ABP is 68% identical to the testis-determining factor, *SRY*, a gene isolated from the sex-determining region on the Y chromosome, and is 98% identical to an autosomal gene that was isolated during the process of screening a whole mouse embryo cDNA library for *SRY*-related sequences. IRE-ABP and the SRY protein share DNA-binding specificity for IRE-A. IRE-ABP binds IRE-A DNA with sequence specificity that overlaps that of the adipocyte IRP-A nuclear extract complex. The sequence in IRE-A DNA that is contacted by IRE-ABP and SRY is identical to the sequence 5′-Py-CTTTG(A/T)-3′, previously defined by Dr. Katherine Jones and her colleagues as a consensus motif contained in several T cell–specific genes that are bound with high affinity by TCF-1α (T cell factor 1α). Thus diverse members of the HMG family of proteins modulate transcription through a similar spectrum of sequences that contain this core motif.

Insulin simultaneously activates processes that result in glucose uptake and utilization and inhibits processes that lead to glucose production. Recent work has explored the possibility that IRE-ABP plays a role in coordinating this adaptive response to a glucose load. Identification of a conserved motif for IRE-ABP provides a way to search for important physiological targets of the IRE-ABP and SRY-like family of transcriptional regulators. The proposed consensus sequence for HMG proteins has been identified in the upstream region of genes that are regulated positively [amylase, the insulin-sensitive glucose transporter 4 (GLUT4)] and negatively [phospho*enol*pyruvate carboxykinase (PEPCK)] by insulin. This laboratory has shown that IRE-ABP footprints these sequences and activates transcription through these sites in cells that do not express IRE-ABP. Thus Dr. Alexander-Bridges and her colleagues conclude that IRE-ABP could bind these sites and regulate these genes *in vivo*.

Insulin regulates GAPDH gene transcription in a tissue-specific manner. Regulation by nutritional manipulations occurs in fat and liver but not muscle. This observation correlates with the fact that IRE-ABP mRNA is expressed in fat and liver, but not muscle, and is induced in the liver of refed rats.

Thus the pattern of IRE-ABP expression is also consistent with its proposed role as a mediator of the adaptive response to glucose. The chronic effect of insulin on the expression of this protein may be mediated at the transcriptional level. An IRE-ABP gene has been isolated to investigate this possibility. It will be interesting to determine whether IRE-ABP gene transcription is subject to autoregulation in response to insulin and/or refeeding.

IRE-ABP Shares Binding Specificity with the Testis-determining Factor

The laboratories of Drs. Peter Goodfellow and Robin Lovell-Badge identified *SRY* as the testis-determining region on the basis of genetic evidence. Several patients with an XY genotype who failed to differentiate to the male phenotype have mutations in the HMG box domain of *SRY*. Certain transgenic mice with an XX genotype that express the cloned *Sry* gene have a male phenotype. Thus this locus was presumed to be the locus responsible for initiation of testis formation in the developing embryo. Because IRE-ABP was isolated on the basis of DNA binding, Dr. Alexander-Bridges and her colleagues were able to demonstrate that derivatives of IRE-ABP or *Sry* that carried mutations associated with sex reversal did not bind DNA.

Rat IRE-ABP showed markedly higher affinity for the IRE-A motif than mouse *Sry*, but the nucleotides contacted by these proteins were essentially identical between these divergent family members. In collaboration with the laboratory of Dr. David Page (HHMI, Massachusetts Institute of Technology),

human SRY and IRE-ABP have been shown to bind IRE-A DNA with equal affinity. Studies in progress will examine derivatives of *SRY* that contain mutations identified in several sex-reversed patients with the goal of defining the spectrum of defects that are associated with sex reversal.

Dr. Alexander-Bridges is also Assistant Professor of Medicine at Harvard Medical School and Clinical Assistant at Massachusetts General Hospital, Boston.

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CELL CYCLE CONTROL

David H. Beach, Ph.D., *Investigator*

The research in Dr. Beach's laboratory is broadly divided into three project areas: 1) cell cycle control in the fission yeast genetic model system, 2) cell cycle control in vertebrate cells, particularly human, and 3) mapping of the fission yeast genome.

Fission Yeast Cell Cycle Control

Single-celled eukaryotes such as the fission yeast and budding yeast continue to provide the most readily accessible material for the discovery of new cell cycle control genes and elaboration of their physiological function. During the past year Dr.

Beach's laboratory has continued to study the so-called cell cycle checkpoints of fission yeast. The division cycle consists of multiple essential events such as DNA replication, nuclear division, and cell division. Checkpoint controls ensure that one cell cycle process does not occur until a prior event is completed. For example, it is essential that cell division not occur until DNA synthesis and repair are fully executed.

Dr. Beach and his colleagues previously identified two genes, *pim1* and *spi1*, that are critically involved in this checkpoint control. The *spi1* gene

encodes a novel low-molecular-weight GTPase, and *pim1* encodes the respective GTP exchange factor. Recent work has shown that the *pim1* product is a highly abundant chromatin protein that is present at approximately one molecule for every four nucleosomes. Cells that lack either *pim1* or *spi1* gene function enter mitosis without completing DNA replication. Thus it is assumed that *pim1/spi1* comprise a chromatin-sensing mechanism that signals the state of the genome to the mitotic regulatory apparatus. Currently the effector mechanism of *pim1/spi1* is being investigated. This pathway involves other genes, specifically *pim2* and *dis2*, that encode the type 1 protein phosphatase.

The G_1 phase of the fission yeast cell cycle is also a subject of investigation. Two new genes, *sdc10* and *cdt1*, have been identified. The *sdc10* gene interacts with the previously known *cdc10* gene to form a cell cycle transcription factor that activates genes required for DNA replication. The most critical of these is *cdt1*, overexpression of which essentially ablates the G_1 phase of the cell cycle. Under some conditions this effect is lethal, because the cell progresses through the division cycle faster than adequate cell growth can occur. The precise biochemical function of the *cdt1* product is unknown and is currently under study.

Mammalian Cell Cycle Control

Dr. Beach's laboratory previously described a new class of cyclins known as D-type cyclins. In humans there are three such cyclins that display striking tissue-specific expression. Cyclin D1, which was independently discovered by several research groups, is also known as PRAD1 or the *Bcl-1* oncogene. To date, cyclin D1 is the only cyclin that has been compellingly linked to human disease. To investigate the possibility that cyclin D2 or D3 might be involved in oncogenesis, Dr. Beach and his colleagues collaborated with Dr. David Ward (Yale University) in mapping the location of these genes in the human genome to 12p13 (D2) and 6p21 (D3). DNA rearrangements at these loci have been implicated in a variety of tumor types, and studies are under way to test whether cyclin D2 or D3 is the active oncogene in these tumors.

Further studies have focused on the biological function of the D-type cyclins. These cyclins associate with at least three protein kinase catalytic subunits (CDK2, 4, and 5) although the relevant protein substrates are presently unknown. Several additional proteins with which D cyclins associate in the cell have been identified. One is a factor that is essential for DNA replication. This observation di-

rectly links the D cyclins with other elements of the cell cycle control pathways and strongly hints that they act at the G_1/S stage of the cycle.

Recently it has become apparent that the tumor-suppressor protein p53 acts as a cell cycle checkpoint regulator. In particular, cells that lack normal p53 function do not arrest in the division cycle in response to damaging agents such as γ irradiation. Although it is likely that p53 acts as a transcription factor, it is not known which gene(s) is the critical target of p53 function. To address this issue Dr. Beach's laboratory again made use of the fission yeast model organism. Overexpression of p53 causes cell cycle arrest in fission yeast, as it does in human cells. Yeast mutants that are resistant to p53 overexpression were generated. In these mutants the p53 protein itself is unaffected, but the cell is relatively unresponsive to p53 cell cycle inhibition. It is hoped that these mutants will lead to the identification of p53 target genes in yeast and thereafter in human cells.

Fission Yeast Genome Mapping

Dr. Beach's laboratory undertook to develop a high-resolution physical map of the 15-Mb genome of the fission yeast. This objective has largely been accomplished: 1,920 cosmid clones have been assembled into ~30 contigs that comprise almost the entire genome. Each of the NotI and Sfi1 sites in the genome have been identified in the cosmids, and ~200 genes have been mapped. The resolution of the genome map presently stands at 10 kb. The existence of the ordered set of fission yeast cosmids renders obsolete the mapping of genes by classical genetic methods.

The fission yeast genome mapping project is supported by funds from the National Institutes of Health.

Dr. Beach is also Senior Staff Scientist at Cold Spring Harbor Laboratory and Adjunct Associate Professor of Microbiology at the State University of New York at Stony Brook.

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MEMBRANE-CYTOSKELETON INTERACTIONS

G. Vann Bennett, M.D., Ph.D., *Investigator*

Dr. Bennett's laboratory has focused on the spectrin-based membrane skeleton, a system of proteins associated with plasma membranes of most animal cells. Spectrin and its associated proteins are candidates to play a role in localization of integral membrane proteins at specialized regions of the plasma membrane. Physiologically important cell domains involving spectrin include axons of neurons, the neuromuscular junction, nodes of Ranvier of myelinated axons, and basolateral domains of epithelial cells.

Research in the past year has addressed basic questions related to association of spectrin with the plasma membrane. Much of the work involves ankyrins, which are a family of spectrin-binding proteins that link the spectrin skeleton to multiple membrane proteins, including ion channels such as the voltage-dependent sodium channel and the Na$^+$,K$^+$ ATPase. Progress in the past year has resulted in the discovery that a major class of ankyrin-binding proteins in adult brain are members of an immunoglobulin (Ig) superfamily of neural cell adhesion molecules, detailed analysis of the structure and regulation of ankyrins, and complete cloning and sequencing of cDNA encoding the β subunit of spectrin.

Brain Ankyrins

Antibodies, cDNA cloning, and mutant mice have been used to distinguish three different ankyrin genes in the nervous system. One ankyrin isoform is localized at nodes of Ranvier and initial axonal segments, sites known to contain the voltage-dependent sodium channel. A specific interaction of this isoform of ankyrin with the sodium channel may play an important role in the morphogenesis and/or maintenance of the node of Ranvier. This ankyrin is likely to represent a distinct gene product, since it persists in mutant mice missing a form of ankyrin located in cell bodies and does not cross-react with antibodies against the major form of brain ankyrin. A research goal has been to identify the gene encoding this nodal/axonal hillock isoform of ankyrin. The cDNAs that encode a new ankyrin closely related to the major isoform of ankyrin in brain have been isolated. The possibility that the new protein represents the form of ankyrin at the node of Ranvier will be evaluated.

The major form of ankyrin in brain occurs as either a 220-kDa polypeptide or a 440-kDa form with a 220-kDa inserted sequence due to alternative splicing of pre-mRNA. The 440-kDa brain ankyrin is the first ankyrin detected in developing brain, with a peak at postnatal day 10 that subsequently falls two- to threefold in adult brain. The 440-kDa neonatal ankyrin is present in neuronal processes including unmyelinated axons and disappears from axons following myelination. This protein thus is a candidate to perform a specialized role in unmyelinated axons. The predicted configuration of the inserted sequence of 440-kDa ankyrin is an ~200-nm random coil. Portions of the inserted sequence that have been expressed in bacteria have such an extended, nonglobular configuration. The 440-kDa ankyrin therefore is shaped like a ball and chain, with membrane and spectrin-binding activities lo-

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cated in the ball and yet-to-be-determined binding sites in the tail. The 440-kDa brain ankyrin is a novel structural protein with the potential to extend from the membrane into the cytoplasm and interact with cytoskeletal elements.

In the future, Dr. Bennett and his colleagues will work to identify the protein(s) in unmyelinated axons that interacts with the 440-kDa ankyrin, particularly the tail domain, and to elucidate the molecular mechanisms regulating transcription of this gene and alternative splicing of its pre-mRNA.

Brain Ankyrin-binding Proteins Related to Neural Cell Adhesion Molecules

A 186-kDa integral plasma membrane protein representing 0.3% of total rat brain membrane protein has been identified as an ankyrin-binding protein and found to be colocalized with ankyrin and coexpressed with the adult form of ankyrin in postnatal development. The sequences of this ankyrin-binding protein, deduced from cDNA clones, and two related polypeptides indicate that these proteins are closely related to neurofascin, a membrane-spanning neural cell adhesion molecule in the Ig superfamily previously implicated in axonal bundling in development of embryonic chicken brain.

The predicted cytoplasmic domains of the rat ankyrin-binding protein and chicken neurofascin are nearly identical and closely related to a group of neural cell adhesion molecules with variable extracellular domains, which include L1, Nr-CAM, and Ng-CAM of vertebrates and neuroglian of *Drosophila*. The ability of ankyrin to bind to neurofascin and possibly to the related cell adhesion molecules may be important in determining the localization of these proteins on the cell surface and in providing a mechanical linkage extending between cells and including cytoplasmic structural proteins. A physiological consequence of convergence of cytoskeletal, transmembrane, and intercellular connections in adult brain may be stabilization of the structure of the nervous system so that this intricate arrangement of cells can survive the traumas of everyday life.

Ankyrin Structure and Regulation

The membrane-binding domains of ankyrins are composed of a tandem series of 33–amino acid repeats that are responsible for recognition of the anion exchanger as well as other targets for ankyrin. The 33-residue repeats are present in a variety of proteins with diverse functions, including regulation of transcription factors, cell cycle regulation, and even neurotoxicity in the case of a spider venom. Functions requiring macromolecular recognition are common functions that ankyrin and these proteins share. A solution to the structure of ankyrin repeats would therefore be useful both in terms of understanding ankyrin function and more generally. As a first step, the basic folding unit of ankyrin repeats has been defined, with the unanticipated result that six repeats are required for a stable globular structure. It should now be possible, in principle, to obtain crystals of the six-repeat unit, which has been expressed in bacteria, and to solve the atomic structure by x-ray crystallography.

An activated ankyrin identified in previous work is missing a 163-residue portion of its regulatory domains because of alternative splicing of pre-mRNA. Recent experiments support the idea that the alternatively spliced segment within the regulatory domain of ankyrin performs a repressor function and acts through an allosteric mechanism involving interaction(s) at a site separate from the binding site for the anion exchanger. These findings provide insight into functional consequences of alternate exon usage and mechanisms of ankyrin regulation.

Dr. Bennett is also Professor of Biochemistry at Duke University Medical Center.

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CONTROL OF TUMOR NECROSIS FACTOR GENE EXPRESSION

Bruce A. Beutler, M.D., *Associate Investigator*

Dr. Beutler's laboratory has continued to focus on the regulation and function of tumor necrosis factor (TNF), a protein originally isolated as a mediator of shock and wasting and also shown to be a selective cytolytic agent capable of destroying a variety of transplantable tumors in animal models. TNF is now known to participate in several inflammatory and catabolic disease processes. Dr. Beutler and his co-workers are seeking to understand not only the pathologic effects of TNF but also the physiologic effects that have justified its phylogenetic conservation. Their method of approach has consisted of 1) an inquiry into the mechanisms by which TNF gene expression is regulated, 2) a determination of the anatomic distribution of biosynthesis *in vivo* under normal conditions, 3) a study of the signaling pathway that leads to TNF biosynthesis in response to endotoxin and other stimuli, 4) an analysis of TNF biosynthesis in endotoxemic animals and in animals exposed to ultraviolet light or to bacterial exotoxins, and 5) an analysis of the effect of a recombinant inhibitor of TNF bioactivity when administered to normal animals or when expressed as the product of a transgene.

TNF biosynthesis is regulated through restriction of TNF gene accessibility, through modulation of TNF gene transcriptional activity, and through modulation of the efficiency with which TNF mRNA is translated. In past studies, Dr. Beutler and his co-workers demonstrated that the TNF promoter/enhancer region responds to inducing stimuli by driving transcription at a more rapid rate, whereas the TNF 3'-untranslated region (UTR), in its mRNA form, responds to inducing stimuli by permitting enhanced translation of the TNF mRNA. The independent action of these two regions of the TNF gene leads to a very high net inducibility of TNF biosynthesis at the protein level. In connection with these studies, it was noted that the TNF promoter is ubiq-uitously active when transfected into mammalian somatic cells; apparently the promoter is well utilized by different tissues.

More recently, the accessibility of the TNF gene was analyzed through somatic cell hybridization experiments. It was determined that the TNF gene is differentially methylated in different cellular environments. In cells that do not express TNF, a highly methylated form of the gene is apparent, and inactivity of the gene seems to be imposed as a dominant trait. Therefore, when the TNF gene in macrophages is moved to a hybrid cell environment through fusion of macrophage and fibroblast cell lines, the normally active TNF locus is silenced. Through the use of reporter constructs, the DNA sequence that is recognized in gene inactivation has been circumscribed to a region 3.2 kb in length, which includes the TNF promoter/enhancer region, the TNF 5'-UTR, and the TNF 3'-UTR.

Gross mutations of the TNF 3'-UTR are known to cause disregulation of TNF biosynthesis. This has been shown in transgenic animals that have received TNF genes in which the 3'-UTR of TNF is supplanted by the 3'-UTR of an unrelated gene. Dr. Beutler and his co-workers have now shown that the 3'-UTR of the TNF gene in mice is relatively polymorphic; some of the mutations detected may have functional consequences related to accessibility or translational control of TNF synthesis.

When the TNF promoter is used to drive the expression of a chloramphenicol acetyltransferase (CAT) coding sequence, which in turn is followed by the TNF 3'-UTR, an excellent mimic of TNF biosynthesis is achieved. Cells transfected with such a reporter construct produce CAT activity under circumstances in which the TNF gene itself is expressed, provided that the latter is accessible. Since CAT remains confined to the cytoplasm of cells in which it is produced, the same reporter construct,

utilized as a transgene, indicates the anatomic locations of TNF biosynthesis in healthy animals and in animals subjected to various types of invasive stimuli or stress.

With the transgene as a guide, it has been possible to demonstrate that TNF is synthesized within the thymus and within the trophoblast of normal mice but in no other tissues examined to date. On the other hand, if mice are injected with bacterial endotoxin, many tissues express the transgene. This provides evidence that numerous cell types, rather than simply cells of lymphoreticular origin, are capable of responding to endotoxin. Moreover, since the transgene seems to be limited in its accessibility much as the authentic TNF gene is, it would seem probable that those tissues that express CAT also express TNF. Immunohistochemical staining of tissues that express CAT activity will hopefully reveal the cell types responsible for TNF biosynthesis within specific tissues.

In addition to endotoxin, other stimuli are known to activate TNF biosynthesis. Ultraviolet (UV) irradiation has been shown to cause TNF expression in keratinocytes. Dr. Beutler and his colleagues have now shown that, unlike endotoxin, which depends on NF-κB elements within the promoter for transduction of its effect on TNF gene transcription, UV light works its effect through very proximal elements within the promoter, only 109 base pairs removed from the cap site. Further mapping studies may reveal the UV-responsive element within the TNF gene.

Predictably, UV irradiation induces expression of the CAT reporter transgene when transgenic mice are exposed to it. Shiga-like toxin, the etiologic agent in the hemolytic uremic syndrome, has been shown specifically to induce expression of the transgene in renal tissue. Thus it would seem feasible to use transgenic mice bearing the CAT reporter gene as a means of identifying signals responsible for tissue-specific TNF synthesis and for charting the expression of TNF *in vivo* in the course of various diseases.

Recombinant inhibitors of TNF bioactivity have been designed and produced in Dr. Beutler's laboratory, to probe the biological function of TNF *in vivo*. The inhibitors are chimeric proteins in which the TNF receptor extracellular domains are covalently linked to an IgG heavy chain. Such inhibitors are of low antigenicity, block the biological activity of TNF more effectively than monoclonal antibodies, and circulate with a long half-life *in vivo*. Preliminary studies indicate that the inhibitors are capable of crossing the placenta and have shown that their administration is compatible with completion of pregnancy. These findings raise questions concerning the function of TNF produced by the trophoblast. Transgenic mice expressing the TNF inhibitors have been produced and may be shown, at term, to express anti-TNF activity in the plasma compartment. Studies of the phenotype of such animals, and in particular of their responses to various disease processes, have now been initiated.

Dr. Beutler is also Associate Professor of Internal Medicine at the University of Texas Southwestern Medical Center at Dallas.

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ADHESION MECHANISMS IN INFLAMMATION AND METASTASIS

Michael P. Bevilacqua, M.D., Ph.D., *Associate Investigator*

Dr. Bevilacqua joined the Howard Hughes Medical Institute in the fall of 1991, after moving his laboratory from Brigham and Women's Hospital at Harvard Medical School to the University of California, San Diego. Prior to this move, he spent nearly eight years studying the role of vascular endothelium in a variety of pathophysiological processes, including inflammation, thrombosis, and tumor cell metastasis. His studies on the mechanisms of adhesion of leukocytes and tumor cells to vascular endothelium led to the identification of two cytokine-inducible endothelial cell surface glycoproteins. The first, originally designated endothelial leukocyte adhesion molecule 1 (ELAM-1), was found to support the interaction of neutrophils, monocytes, and some lymphocytes with activated vascular endothelium. Characterization of this molecule revealed a type 1 transmembrane protein with an amino-terminal lectin-like domain, an epidermal growth factor (EGF) repeat, and six complement regulatory-like repeats. Two other molecules cloned contemporaneously with ELAM-1 were found to have a similar domain composition, thereby establishing a new family, now called selectins.

The second molecule identified in Dr. Bevilacqua's laboratory was called inducible cell adhesion molecule 110 (INCAM-110; also known as VCAM-1). INCAM-110 can support the adhesion of lymphocytes and monocytes (but not neutrophils) as well as nonlymphoid tumor cells, including melanomas. This molecule is expressed by activated endothelium and by dendritic cell populations in lymphoid tissues and skin.

Quantitative Assessment of Selectin-Carbohydrate Interactions

The selectins act in concert with other molecules to support adhesion of leukocytes to the blood vessel wall—a key step in the inflammatory response to injury and infection. The term selectin was originally proposed to highlight the presence of the lectin domain and to emphasize the selective nature of the expression and function of these molecules. A standard nomenclature was agreed on that designates each family member according to the cell type on which it was originally identified: E-selectin (previously ELAM-1), endothelium; P-selectin, platelets; and L-selectin, leukocytes.

Dr. Bevilacqua has studied the ability of solution-phase synthetic oligosaccharides to block E-, P-, and L-selectin–dependent interactions in three assays of increasing biological complexity: 1) a competitive ELISA involving direct binding of selectin-immunoglobulin (selectin-Ig) fusion proteins to carbohydrate-bearing substrates, 2) a cell-protein adhesion assay using immobilized selectin-Ig, and 3) a cell-cell adhesion assay with cytokine-activated human endothelial cells.

E- and P-selectin-Ig fusion proteins bound to immobilized bovine serum albumin (BSA)-neoglycoproteins containing sialyl Lewis x (sLe^x; Neu5Acα2-3Galβ1-4[Fucα1-3]GlcNAc) or sialyl Lewis a (sLe^a; Neu5Acα2-3Galβ1-3[Fucα1-4]GlcNAc) in a Ca^{2+}-dependent manner. Solution-phase sLe^x tetrasaccharide blocked this interaction by 50% at a concentration ≈ 750 μM (IC$_{50}$). With an IC$_{50} \approx 220$ μM, sLe^a was approximately threefold more effective. Nonsialylated, nonfucosylated derivatives showed little or no activity at concentrations up to 1 mM. Attachment of an 8-methoxycarbonyloctyl aglycone in a β linkage to the anomeric carbon of the GlcNAc of sLe^x or sLe^a increased their blocking activity nearly twofold. In addition, replacement of the 2-*N*-acetyl substituent of the GlcNAc by an azido or amino group resulted in substantial increases in activity, with the most potent inhibitor being amino-substituted sLe^a, which was 36-fold more active (IC$_{50} \approx 21$ μM) than the reducing tetrasaccharide sLe^x. In contrast to results obtained with E-selectin-Ig, P-selectin-Ig binding to immobilized BSA-sLe^a

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was blocked modestly by most oligosaccharides at 1 mM. IC_{50} values of soluble oligosaccharides determined in competitive binding studies accurately predicted blocking of leukocyte adhesion to recombinant E- and P-selectin-Ig and to cytokine-activated endothelium. L-selectin-Ig demonstrated little binding to immobilized BSA-sLe^a or BSA-sLe^x, precluding evaluation in competitive ELISAs.

Together these results indicate that the three selectins have distinct carbohydrate recognition characteristics and that specific modifications of the oligosaccharides sLe^x and sLe^a can result in higher affinity ligands for E-selectin. Moreover they suggest avenues for the development of novel anti-inflammatory therapeutic agents.

Cell Adhesion Molecules in Lung Injury and Inflammation

To assess the role of selectin-carbohydrate interactions *in vivo*, Dr. Bevilacqua and his colleagues are using murine models of acute lung injury. Intratracheal injection of lipopolysaccharides and inflammatory cytokines was shown to induce leukocyte extravasation and movement into the airspace. Neutrophils recovered in bronchoalveolar lavage fluid increase dramatically during the first hours postinjection, peaking between 6 and 24 hours. This is followed by an exudation of monocytes and lymphocytes that reaches a maximum between 24 and 48 hours. In an attempt to identify potential anti-inflammatory agents, Dr. Bevilacqua and his colleagues are evaluating selected carbohydrates. In preliminary studies, the tetrasaccharides sLe^x and sLe^a given intravenously at 10 mg/kg blocked neutrophil exudation by ~50%. In combination, sLe^x and sLe^a were more potent than either compound alone. Significant blocking activity was achieved with a total dose of 200 mg of oligosaccharide per mouse. Assuming a blood volume of 2 ml, this amount would yield a maximal blood concentration of 100 μM. These compounds appear to be more effective (i.e., work at lower doses) in blocking leukocyte extravasation *in vivo* than they are in blocking leukocyte adhesion *in vitro*.

Endothelial Adhesion Molecules in Metastasis

Since joining the Howard Hughes Medical Institute, Dr. Bevilacqua and his colleagues have expanded their efforts in the study of endothelial adhesion molecules in metastasis. Their recent efforts have focused primarily on the adhesion and metastasis of colon cancer cells. Nearly all human colon cancer cell lines tested demonstrate enhanced binding to cytokine-activated endothelium, a process that is blocked by anti–E-selectin antibodies. Similarly, these cells bind to COS cells transfected with recombinant E-selectin, as well as to dishes coated with E-selectin-Ig. One cell line, CaCo2, demonstrates little or no E-selectin–dependent adhesion. This pattern of adhesion accurately predicts the metastatic potential of these tumor cell lines in a murine model. Treatment of mice with the inflammatory cytokine interleukin-1 results in a 3- to 10-fold increase in pulmonary metastatic nodules after intravenous injection of all the colon cancer lines tested except CaCo2. Present efforts are focused on characterization of the tumor cell surface carbohydrate ligands for E-selectin and evaluation of potential inhibitors of metastasis. Separate studies focus on the interaction of cancer cells with P- and L-selectin.

Preliminary studies on acute lung injury and the research on endothelial adhesion molecules in metastasis have been supported in part by funds from the Pew Foundation.

Dr. Bevilacqua is also Associate Professor of Pathology and Member of Cellular and Molecular Medicine at the University of California, San Diego, and a Pew Scholar in the Biomedical Sciences.

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MOLECULAR MECHANISMS OF INSULIN AND POLYPEPTIDE GROWTH FACTOR ACTION

Perry J. Blackshear, M.D., D.Phil., *Investigator*

Dr. Blackshear's laboratory is interested in the molecular mechanisms of action of insulin and other polypeptide hormones and growth factors. In the past several years, these studies have focused on two major areas: insulin's regulation of protein biosynthesis and the involvement of protein kinase C substrates in agonist-signaling pathways involving this family of protein kinases.

The studies of insulin-stimulated protein biosynthesis involve one example of stimulated mRNA translation and two examples of rapid stimulated gene transcription. Studies by Dr. Joyce Manzella in the laboratory showed that insulin could rapidly stimulate the biosynthesis of the enzyme ornithine decarboxylase (ODC), primarily at the level of mRNA translation. She further showed that the insulin stimulation of ODC biosynthesis appeared to involve the unwinding or melting of mRNA secondary structure, permitting translation to proceed more rapidly. This effect was particularly striking in the case of the ODC mRNA, since the extreme 5' portion of the long ODC mRNA leader sequence contains a large stem-loop structure of pronounced negative free energy. The mechanism by which insulin stimulated this mRNA melting activity appeared to involve the rapid stimulated phosphorylation of initiation factors eIF4B and 4F, both of which are involved in either cap binding or unwinding of mRNA secondary structure. In this way, insulin and other growth factors can preferentially stimulate the translation of mRNAs with intense secondary structure in their 5'-untranslated region.

Dr. Blackshear and his colleagues have also continued to work on the rapid induction by insulin of certain early response genes, including the c-*fos* proto-oncogene and a gene encoding a zinc finger protein of no known function. In previous studies with the c-*fos* promoter, the major insulin response element was localized to a promoter segment known as the serum response element (SRE). More recent studies by Dr. Rajesh Malik showed that insulin and other growth factors stimulated formation of two different ternary complexes involving the SRE within 2 min of hormonal stimulation. Current studies in the laboratory involve purification of the proteins involved in these complexes, their molecular cloning, and the elucidation of the covalent modification that leads to their increased binding to the c-*fos* SRE.

The other major area under study in the laboratory involves the stimulated phosphorylation by protein kinase C (PKC) of a small family of substrate proteins related to the myristoylated alanine-rich C-kinase substrate (MARCKS) protein. One recent study involved an exploration of the factors responsible for the binding of this myristoylprotein to cellular membranes. Dr. Daniel George in the laboratory found that the myristoylated protein bound to cell membranes much more rapidly than did a non-myristoylated mutant protein. However, this membrane association did not appear to involve binding to a specific membrane "receptor" protein, as has been shown for another myristoylprotein, but instead seems to involve hydrophobic interactions between the fatty acid moiety and the membrane lipids.

Other studies are attempting to prove that the high-affinity binding of MARCKS to calmodulin, which occurs readily *in vitro*, also occurs *in vivo* at normal ambient calcium concentrations. Dr. Robert D. Hinrichsen and Dr. Blackshear showed that MARCKS calmodulin-binding peptides, when injected into *Paramecium*, could elicit a behavioral response in these organisms that is typical of decreased calmodulin concentrations, suggesting strongly that the MARCKS peptide was interacting with calmodulin and preventing the normal behavioral response. Such a response was not seen with mutant peptides that had lower affinity for calmodulin and could be reversed by PKC-stimulated phosphorylation of the peptide, as occurs in cell-free systems. These studies demonstrated that MARCKS calmodulin-binding peptides can actually bind calmodulin at ambient cellular calcium concentrations and that this binding can be reversed by PKC-dependent phosphorylation of MARCKS peptide and proteins.

Another group of studies involved the elucidation of the structure and function of the MRP protein, a recently discovered MARCKS homologue. This protein is homologous to the MARCKS protein in the three regions in which the MARCKS proteins from different animal species are conserved: the extreme amino-terminus myristoylation consensus sequence, the site of splicing of the single intron, and the basic phosphorylation site/calmodulin-binding domain. Studies by George Verghese in the laboratory identified this protein as a myristoylprotein both *in vivo* and *in vitro*; as a high-affinity phosphorylation substrate for PKC; and as a high-affinity calmodulin-binding protein, with disruption of the complex occurring after PKC-dependent phosphor-

ylation. It is, however, distinct from the MARCKS protein in that it is encoded by a completely different gene and shows differences in developmental and tissue-specific expression.

The cellular functions of both the MRP and MARCKS proteins are unknown, although attributes include calmodulin binding and probably actin binding. Studies under way to elucidate functions for the proteins include microinjection of antibodies to the proteins in intact cells; the use of antisense DNA to inhibit the biosynthesis of these proteins; and, ultimately, the use of homologous recombination to disrupt the endogenous genes encoding these proteins and the evaluation of the resulting phenotypes. A number of chimeric mice have already been created for the MARCKS gene, and their offspring are being evaluated for targeted disruption of one endogenous allele. At the same time, disruption constructions are being prepared for the MRP gene, which was cloned and sequenced by Dr. David Lobach in the laboratory earlier this year. The goal of these studies will be the formation of mice, and cell lines derived from them, completely defective in the MARCKS and MRP genes; these animals and cells should be useful in determining the function of these proteins in normal physiology.

Dr. Blackshear is also Professor of Medicine and Professor of Biochemistry at Duke University Medical Center.

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PROTEIN TRAFFIC ACROSS INTRACELLULAR MEMBRANES

GÜNTER BLOBEL, M.D., PH.D., *Investigator*

Most cellular membranes contain systems for the unidirectional translocation of proteins across these membranes. The nuclear envelope is unique among cellular membranes in that it contains an organelle, the nuclear pore complex (NPC), that allows bidirectional transport, in and out of the nucleus, not only of proteins but also of RNPs and most likely also of DNPs. Most of the research efforts of Dr. Blobel and his colleagues are aimed at a detailed molecular characterization of these transport processes.

For unidirectional translocation, protein-conducting channels (PCCs) are involved. These PCCs can open to an estimated diameter of 2–3 nm. Therefore proteins can be translocated only in an unfolded configuration. In addition to their ability to open and close across the membrane, PCCs can also open and close in a second dimension, toward the bilayer, and therefore effect integration of proteins into the lipid bilayer.

NPCs are huge transporters (25 times the mass of a ribosome) that can open to a diameter of ~25 nm. Transport therefore does not require unfolding. Unlike PCCs, NPCs cannot integrate proteins into the bilayer.

Protein-conducting Channel is Gated Open by Signal Sequence

In 1990, Drs. Sanford Simon and Blobel used electrophysiological methods to show the existence of a PCC in the mammalian endoplasmic reticulum. Mammalian rough microsomal vesicles were fused to a planar bilayer. Each rough microsomal vesicle contains dozens of attached ribosomes in the process of translocating a polypeptide chain across the membrane. Therefore each microsomal vesicle contains dozens of PCCs. These PCCs were found to be electrophysiologically silent, i.e., impermeable to ion fluxes when occupied by translocating chains.

However, after evacuation of the translocating chains by ribosome-mediated coupling to puromycin, large channels, each of a conductance of 220 pS at 50 mM KCl, could be detected. These channels closed abruptly when the ribosome was removed at high KCl concentrations.

In the past year, Drs. Simon and Blobel investigated how these large PCCs would be opened. Could the PCC be a ligand-gated channel, i.e., opened by the signal sequence of the translocating polypeptide as predicted in the signal hypothesis? To answer this question a signal peptide was chemically synthesized and tested in a planar bilayer system, this time containing fused vesicles of the *Escherichia coli* plasma membrane. Addition of the signal peptide in subnanomolar concentrations specifically to the cytoplasmic side of the fused vesicle membrane revealed channels of similar conductance, 220 pS at 50 mM KCl, as seen in mammalian rough microsomes. These data indicated that PCCs are indeed signal-sequence gated. Thus the principal function of the various membrane-specific signal sequences is to open cognate PCCs in intracellular membranes.

Transport on Intranuclear Tracks

Another seminal discovery of the past year was the detection by Drs. Thomas Meier and Blobel of intranuclear tracks, up to several microns in length, on which transport between nucleoli and a small number of dedicated NPCs appears to occur. These tracks were detected through immunolocalization of Nopp140 (nucleolar phosphoprotein of 140 kDa). Drs. Meier and Blobel showed that Nopp140 shuttles between the nucleolus and the cytoplasm. Immunoelectron microscopy revealed that Nopp-140 was localized in striking curvilinear arrays that extended from the nucleolus to some of the NPCs. Could these tracks be actin filaments? And if so, would S_1-type myosin motors move cargo (such as Nopp140) on these tracks? Are all of the intranuclear transcription and RNP assembly sites connected by tracks to NPCs? Are these tracks reversibly disassembled during mitosis? These are only a few questions elicited by these findings. The answers are bound to affect profoundly current thinking about nuclear architecture and processes such as transcription, DNA replication, and mitosis.

Two Distinct Cytosolic Fractions for Protein Targeting and Translocation through the Nuclear Pore Complex

Drs. Mary Moore and Blobel have identified two cytosolic fractions from *Xenopus* oocytes that contain the activity necessary to support both steps of nuclear import in digitonin-permeabilized mammalian cells: binding at the nuclear envelope and translocation through the NPC. The first cytosolic fraction (fraction A) interacts with an import-competent, but not a mutant, nuclear localization sequence-bearing conjugate and stimulates its accumulation at the nuclear envelope in an ATP-independent fashion. The second cytosolic fraction (fraction B) gives no discernible effect when added alone; but when added together with fraction A, or after fraction A, it stimulates the passage of the conjugate from the outer nuclear envelope to the interior of the nucleus in an ATP-dependent fashion.

Dr. Blobel is also Professor of Cell Biology at the Rockefeller University.

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MOLECULAR BIOLOGY OF THE EXTRACELLULAR MATRIX

JEFFREY F. BONADIO, M.D., *Assistant Investigator*

The long-term goal of Dr. Bonadio's research effort is to understand how the extracellular matrix contributes to the structure and function of tissues. The extracellular matrix evolved to protect cells and hold them in spatial arrangements required for anatomical and physiological functions. The extent and organization of the matrix at different anatomical sites is a reflection of the specialized functions of cells and their interactions with the environment. In the adult, the matrix plays a role in wound healing and contributes to the ability of organs to bear mechanical loads, e.g., as occur in the musculoskeletal system during locomotion, the uterus during parturition, the lung during respiration, and the cardiovascular system during circulation.

Dr. Bonadio's laboratory is investigating the molecular basis of load bearing in the mammalian skeleton. Type I collagen is particularly abundant in skeletal tissues and may represent a primary load-bearing element. Dr. Bonadio and his colleagues initially sought to define the mechanical role of collagen by creating genetic deletions in the germline of mice. *Mov* mouse strains were generated in the laboratory of Dr. Rudolf Jaenisch by exposing mouse embryos to the Moloney murine leukemia virus. In *Mov13* mice the retrovirus integrated within the first intron of the $\alpha1(I)$ collagen gene. With the exception of embryonic odontoblasts and a small subpopulation ($\sim5\%$) of osteoblasts, the proviral insert prevents initiation of transcription at the collagen locus. Mice homozygous for the null mutation produce no type I collagen and die *in utero* because of the decreased structural integrity of cardiovascular tissues. In heterozygous *Mov13* mice, the block in transcription initiation is associated with a 50% reduction in type I collagen production by connective tissue cells and a 50% decrease in tissue collagen content. Dr. Bonadio and his colleagues found that the collagen deficiency is associated with hearing loss and with morphological and functional connective tissue defects.

A subsequent analysis of the transgenic mouse strain *Mov13* included a successful attempt to rescue the collagen-deficient phenotype. These experiments argue convincingly that type I collagen normally contributes to the relative ductility of cortical bone, an important property that allows repeated loading without macroscopic failure. By contributing to ductility, type I collagen allows bone to be used repeatedly and yet avoid macroscopic failure: following the application of a large mechanical load, bone will plastically deform before failure because of its collagen content. Type I collagen is highly conserved in evolution, and one reason may be the fundamental contribution it makes to the material properties of the skeleton. The latter observation was dramatically underscored by the fact that the *Mov13* phenotype was successfully complemented by the human $\alpha1(I)$ collagen gene.

Dr. Bonadio's laboratory currently is attempting to generate a mouse strain deficient in fibrillin, an extracellular matrix molecule that participates in the formation of ubiquitous 10- to 12-nm microfibrils. Members of the laboratory have cloned and characterized the full-length cDNA sequence of a mouse fibrillin gene. Preliminary evidence suggests that there are at least two other members of the mouse fibrillin gene family. With colleagues the laboratory is working to generate fibrillin-deficient mouse strains by targeted disruption of the genetic locus, using the technique of homologous recombination in embryonic stem cells. If this work is successful, founder mice will then be used to breed mutant strains that are either homozygous or heterozygous for the nonfunctional fibrillin allele. The mutant mice will be analyzed in a manner similar to *Mov13* mice. This project was supported in part by a grant from the National Institutes of Health.

Dr. Bonadio and his colleagues recently demonstrated that small changes in skeletal geometry can lead to a significant improvement in mechanical function. Over a two-month period, cortical bone cells of the mouse femur were stimulated to synthesize, deposit, and mineralize a small amount of new bone along periosteal surfaces. The deposition of new bone increased cross-sectional geometry, and this increase in turn led to a dramatic increase in strength. This work suggests that regulation of the pattern of gene expression in osteogenic cells may represent a rational approach to treating skeletal fragility, an important problem in industrialized countries. The goal of this therapy would be to strengthen long bone by altering its geometry according to a rational design. This project was supported in part by a grant from the National Institutes of Health.

The laboratory has sought to test the validity of this therapeutic strategy in animal model systems. Toward this end the laboratory has identified several candidate genes whose products will stimulate the metabolism of periosteal bone cells, and the appropriate gene transfer constructs have been prepared.

Finally, Dr. Bonadio's laboratory has developed a novel, *in vivo* gene transfer method. Along with colleagues at the Hillenbrand Biomedical Engineering Center of Purdue University, members of the laboratory have been able to transfer and express plasmid and retroviral DNAs directly into the cells of regenerating tendon, ligament, cartilage, and bone. This discovery represents the first successful *in vivo* gene transfer experiment in skeletal tissues, and it provides the first indication that gene therapy is possible for skeletal disorders. It will also allow the laboratory to explore the biological mechanisms that govern the tissue regeneration.

Dr. Bonadio is also Assistant Professor of Pathology at the University of Michigan Medical School.

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MOLECULAR STUDIES OF CALCIUM CHANNELS AND THE DYSTROPHIN-GLYCOPROTEIN COMPLEX

KEVIN P. CAMPBELL, PH.D., *Investigator*

Dr. Campbell's research is aimed at understanding the structure and function of the membrane proteins involved in regulating intracellular Ca^{2+} in excitable cells. His laboratory has focused on the identification, purification, and characterization of the Ca^{2+} channels that function in muscle excitation-contraction coupling and in neuronal Ca^{2+} homeostasis. In addition, his laboratory has studied the structure and function of dystrophin and associated membrane glycoproteins, to develop an understanding of why the absence of dystrophin results in muscular dystrophy.

Intracellular Ca^{2+} Release Channels in Muscle and Neurons

One goal of Dr. Campbell's research is to understand the structure and function of intracellular Ca^{2+} release channels. In skeletal muscle, Ca^{2+} release from the sarcoplasmic reticulum (SR) initiates muscle contraction. Dr. Campbell's laboratory previously showed that the purified ryanodine receptor from skeletal muscle is identical to the SR Ca^{2+} release channel and SR "feet" structures. In the past year the laboratory has determined that abnormalities in the regulation of Ca^{2+} release that had been found in skeletal muscle from humans and pigs with malignant hyperthermia are directly due to a mutation in the ryanodine receptor. Previously the labo-

ratory had found that tryptic digestion of SR vesicles produced clear and reproducible differences between the immunostaining pattern of the malignant hyperthermic and normal ryanodine receptor peptides. In the past year the laboratory has shown that in isolated membranes the amino acid change in the ryanodine receptor from Arg^{615} to Cys^{615} is directly responsible for the altered tryptic peptide map, due to the elimination of the Arg^{615} cleavage site. These results suggest that the 86- to 99-kDa domain of the ryanodine receptor containing residue 615 is near the cytoplasmic surface of the receptor and likely near important Ca^{2+} channel regulatory sites.

Dr. Campbell's research also concerns ryanodine receptor/Ca^{2+} release channels in neuronal cells and nonexcitable cells. Previously his laboratory showed that the brain ryanodine receptor functions as an intracellular Ca^{2+} release channel and that it is the probable gating mechanism for caffeine- and Ca^{2+}-sensitive Ca^{2+} stores in neurons. In the past year a release channel in sea urchin eggs has been identified. Immunolocalization of the Ca^{2+} release channel reveals a cortical reticulum or "honeycomb" staining network that surrounds cortical granules and is associated with the membranes of the cortical endoplasmic reticulum. An ~380-kDa protein of sea urchin egg cortices is identified by immunoblot analysis with ryanodine receptor anti-

bodies. These results suggest the presence of a ryanodine-sensitive Ca^{2+} release channel within the sea urchin egg cortex. In the upcoming year studies of this receptor should provide insights into its role in Ca^{2+} homeostasis in excitable and nonexcitable cells and its distribution within the central nervous system.

Voltage-Sensitive Ca^{2+} Channels in Neurons

A second major area of research concerns voltage-sensitive Ca^{2+} channels in excitable cells. The dihydropyridine receptor (DHPR), previously purified in Dr. Campbell's laboratory from rabbit skeletal muscle, consists of four subunits (α_1, α_2, β, and γ). In neurons, voltage-sensitive Ca^{2+} channels exist as several types (L, N, T, and P) with different kinetic and pharmacological properties. Dihydropyridines bind specifically to L-type Ca^{2+} channels and alter their channel activity. For N-type channels, which are likely responsible for triggering neurotransmitter release at synapses, ω-conotoxin is largely specific.

In the past year, Dr. Campbell's laboratory has used antibodies and cDNA probes to the various subunits of the dihydropyridine-sensitive channels of skeletal muscle to study N-type Ca^{2+} channels (ω-conotoxin sensitive) in neurons. Antibodies against the subunits of the dihydropyridine-sensitive L-type Ca^{2+} channel were tested for their ability to immunoprecipitate the high-affinity ($K_d = 0.13$ nM) ^{125}I-ω-conotoxin GVIA receptor from rabbit brain membranes. Monoclonal antibody VD2$_1$ against the β subunit of the DHPR specifically immunoprecipitated up to 86% of the ^{125}I-ω-conotoxin receptor, whereas specific antibodies against the α_1, α_2, and γ subunits did not precipitate the brain receptor. The ω-conotoxin receptor immunoprecipitated by monoclonal antibody VD2$_1$ showed high-affinity ^{125}I-ω-conotoxin binding, which was inhibited by unlabeled ω-conotoxin and by $CaCl_2$ but not by various dihydropyridines. These results suggest that the brain ω-conotoxin–sensitive Ca^{2+} channel contains a component homologous to the β subunit of the dihydropyridine-sensitive Ca^{2+} channel of skeletal muscle and brain. Work is now in progress to purify the ω-conotoxin–sensitive Ca^{2+} channel, using affinity chromatography to analyze its subunit composition and to demonstrate that it is the N-type Ca^{2+} channel.

A cDNA clone encoding a protein with high homology to the β subunit of the rabbit skeletal muscle dihydropyridine-sensitive Ca^{2+} channel was also isolated. This rat brain β-subunit cDNA hybridizes to a 3.4-kb message that is expressed in high levels in the cerebral hemispheres and hippocampus but is significantly reduced in cerebellum. The open reading frame encodes 597 amino acids with a predicted mass of 65,679 Da and is 82% homologous with the skeletal muscle β subunit. The brain cDNA encodes a unique 153–amino acid carboxyl terminus and predicts the absence of a muscle-specific 50–amino acid internal segment. It also encodes numerous consensus phosphorylation sites, suggesting a role in Ca^{2+} channel regulation. Thus the encoded brain β subunit, which has a primary structure highly similar to its isoform in skeletal muscle, may have a comparable role as an integral regulatory component of a neuronal Ca^{2+} channel. A grant from the National Institutes of Health provided support for the project described above.

Dystrophin-Glycoprotein Complex

Dystrophin, the high-molecular-weight protein product of the Duchenne muscular dystrophy (DMD) gene, is localized to the sarcolemma of normal skeletal muscle but is absent from the skeletal muscle of DMD patients and *mdx* mice. The predicted amino acid sequence of dystrophin suggests that dystrophin is a membrane cytoskeletal protein involved in the anchoring of sarcolemmal proteins to the underlying cytoskeleton. Dr. Campbell and his colleagues discovered the dystrophin-glycoprotein complex, a large oligomeric complex containing dystrophin, a 59-kDa protein triplet, and four sarcolemma glycoproteins of 156, 50, 43, and 35 kDa. In the past year the membrane organization of this complex, including the stoichiometry, cellular location, glycosylation, and hydrophobic properties of the components, has been determined. The 156-, 59-, 50-, 43-, and 35-kDa dystrophin-associated proteins (DAPs) each possess unique antigenic determinants, enrich quantitatively with dystrophin, and were localized to the skeletal muscle sarcolemma. The 156-, 50-, 43-, and 35-kDa DAPs contain Asn-linked oligosaccharides. The 156-kDa dystrophin-associated glycoprotein (DAG) contained terminally sialylated Ser/Thr-linked oligosaccharides. Dystrophin, the 156-kDa, and the 59-kDa DAPs are peripheral membrane proteins; the 50-, 43-, and 35-kDa DAGs and the 25-kDa DAP are integral membrane proteins. Thus dystrophin and its 59-kDa associated protein are cytoskeletal elements that are tightly linked to a 156-kDa extracellular glycoprotein by a complex of transmembrane proteins. The membrane organization of the dystrophin-glycoprotein complex and the high density of dystrophin in the sarcolemma membrane suggest that this complex could have an important structural role in skeletal muscle.

In the past year Dr. Campbell's group also exam-

ined the subcellular localization and biochemical properties of dystrophin-related protein (DRP), an autosomal gene product with high homology to dystrophin. DRP is enriched in isolated sarcolemma from control and *mdx* mouse muscle but is much less abundant than dystrophin. Immunofluorescence microscopy localized DRP almost exclusively to the neuromuscular junction region in adult rabbit and mouse skeletal muscle, as well as *mdx* mouse muscle and denervated mouse muscle. Thus DRP is a neuromuscular junction–specific membrane cytoskeletal protein that may play an important role in the organization of the postsynaptic membrane of the neuromuscular junction.

Dystroglycan, a Novel Laminin-binding Protein

Because the abnormal expression of the DAGs may play a crucial role in the molecular pathogenesis of DMD, it was essential for Dr. Campbell's laboratory to identify their normal function. A major emphasis in the laboratory is now directed toward the isolation of cDNA clones for each DAP. The primary structures of two components of the dystrophin-glycoprotein complex, the 43-kDa DAG and 156-kDa DAG (dystroglycan), have been determined. Sequence analysis of cDNAs reveals an open reading frame encoding a 97-kDa precursor polypeptide with no significant sequence similarity with any known proteins. The amino-terminal portion of the precursor polypeptide is processed into the mature 156-kDa DAG with a putative protein core of ~56 kDa with potential attachment sites for O-linked carbohydrates. The carboxyl-terminal portion of the precursor polypeptide is processed into the mature 43-kDa DAG with potential *N*-glycosylation sites, a single transmembrane domain, and a 120–amino acid long cytoplasmic tail. Northern and Western blot analyses have demonstrated that the 43- and 156-kDa DAGs are expressed in both muscle and nonmuscle tissues. The specific mRNA for the 43- and 156-kDa DAG is expressed at normal amounts in *mdx* and DMD skeletal muscle, whereas both glycoproteins are greatly reduced in dystrophin-deficient muscle. Functional studies have demonstrated that dystroglycan binds laminin, a well-characterized component of the extracellular matrix.

Thus the results demonstrate that dystroglycan is a novel laminin-binding glycoprotein and suggest that the function of the dystrophin-glycoprotein complex is to link the subsarcolemmal cytoskeleton to the extracellular matrix. The goal for the next year is to clone the other DAPs in order to express the entire complex in nonmuscle cells to study its function and laminin-binding properties.

Molecular Pathogenesis of Muscular Dystrophy

Characterization of the status of DAPs in dystrophin-deficient muscle was necessary to understand the molecular pathogenesis of DMD. The relative abundance of each component of the dystrophin-glycoprotein complex in skeletal muscle from normal and *mdx* mice, which are missing dystrophin, was examined last year. Immunoblot analysis using total muscle membranes from control and *mdx* mice (ages 1 d to 30 wk) found that all of the DAPs were greatly reduced (80–90%) in *mdx* mouse skeletal muscle. The specificity of the loss of the DAPs was demonstrated by the finding that the major glycoprotein composition of skeletal muscle membranes from normal and *mdx* mice is identical.

Immunofluorescence microscopy confirmed these results and showed a drastically reduced density of DAPs in *mdx* muscle cryosections compared with normal and *dy/dy* mouse muscle. Therefore all of the DAPs are significantly reduced in *mdx* skeletal muscle, and the loss of these proteins may be due to the absence of dystrophin and not to secondary effects of muscle fiber degradation. In the next year these studies will be extended to DMD patients. In addition, DAP status will be examined in various autosomal muscular dystrophy patients. A grant from the Muscular Dystrophy Association provided partial support for the project described above.

Dr. Campbell is also Professor of Physiology and Biophysics at the University of Iowa College of Medicine, Iowa City, and University of Iowa Foundation Distinguished Professor of Physiology and Biophysics.

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GENETIC CONTROL OF PATTERN FORMATION IN *DROSOPHILA* AND OTHER INSECTS

Sean B. Carroll, Ph.D., *Assistant Investigator*

During animal development, cells of the growing
embryo become organized into structures of widely
varying number, size, shape, composition, and
function. Genetic and embryological studies of a
model animal, the insect *Drosophila melanogas-
ter,* have shown that the specification of the overall
body plan, the formation of complex arrays of tis-
sues and organs, and the assignment of individual
cell fates occur in a highly ordered temporal se-
quence and spatial pattern. Genetic screens for pat-
tern-disrupting mutations have identified many of
the loci that influence the size, shape, number, and/
or function of body structures and have catalyzed an
integrated molecular and embryological approach
to understanding the control and function of pat-
tern-regulating genes and the overall logic of devel-
opmental processes. More recently, the scope of this
"molecular embryology" has widened toward the
analysis of the evolution of developmental pro-
cesses in diverse animals, with the goal of identify-
ing common themes in body design and understand-
ing the genetic basis of morphological evolution.

Research is currently centered on the cell biology
and genetics of pattern formation in animals at three
levels. The first involves the formation of large-scale
patterns in *Drosophila* and, more specifically, the
regulation and function of the more globally acting
genes that establish segment number and segmental
pattern in the larva and control appendage forma-
tion in the adult. The second level addresses pattern
formation on a finer scale within segments and ap-
pendages, using both the *Drosophila* nervous sys-
tem and, more recently, butterfly wing color pat-
terns as models. The third level is broadly
concerned with the molecular and developmental
basis for the evolution of both global and fine-scale
patterns among insects.

Pair-rule Genes Integrate Segment Formation with Intrasegmental Pattern Formation

The first sign of the future segmental organization
of the *Drosophila* embryo is the expression of the
pair-rule genes in alternating stripes encircling the
early blastoderm embryo. Two fundamental ques-
tions concerning the regulation and function of
these genes are of the most interest: 1) How are
these periodic patterns of gene expression initially
established in the embryo? 2) How do the pair-rule
genes control the subsequent segmental organiza-
tion of the developing embryo?

Using the *hairy* pair-rule gene as a model for the
regulation of periodic gene expression, several labo-
ratories have shown that the aperiodically expressed
gap genes establish the striped pattern of *hairy* ex-
pression. Each *hairy* stripe is regulated by separable
cis-acting regulatory sequences upstream of the
hairy gene. Jim Langeland has shown that the linear
order of the cis-acting regulatory sequences is con-
served in distant *Drosophila* species (*D. virilis*) and
that all of the gap proteins appear to regulate the
same stripe boundaries. Even more striking was the
finding that these *D. virilis* sequences, when trans-
formed into *D. melanogaster*, are capable of driv-
ing the normal pattern of seven *hairy* stripes. By
localizing stretches of conserved regulatory se-
quence, a minimal regulatory element for one *hairy*
stripe has been identified that is repressed by two

gap proteins and activated by a general transcription factor.

The pair-rule gene products regulate overall segmental pattern by controlling the initial expression of the segment polarity loci, a large group of genes that are usually expressed in one band of cells within each segment. James Skeath has shown that other genes involved in the formation of structures within segments are also regulated by pair-rule genes. The proneural genes *achaete* and *scute*, for example, are expressed in several anteroposterior rows of ectodermal cell clusters from which individual neuroblasts will segregate. The registration of these rows is achieved by combinations of pair-rule gene products directly regulating proneural gene activity. Thus global (segmentation) and fine-scale (intrasegmental) pattern formation are integrated by a common set of regulatory genes.

To dissect the molecular regulatory mechanisms underlying the global control of *achaete* and *scute* gene expression, Jim Skeath, Dr. Grace Panganiban, and Jane Selegue have isolated certain cis-acting regions that regulate both genes simultaneously. This common intergenic region (>15 kb in size) is necessary for spatial regulation by the pair-rule genes. Different sets of pair-rule gene products establish at least two different anteroposterior domains of *achaete* and *scute* expression. Along the dorsoventral axis, other regulatory proteins restrict proneural gene activity to the ventral neurogenic region. Thus the initial two-dimensional pattern of proneural cell clusters expressing *achaete/scute* is carved out by the combined action of two sets of axis-patterning genes acting through a common regulatory region.

A Genetic Hierarchy Guides Wing Formation

One of the least well understood aspects of animal development is the formation of the various adult limbs and appendages. In *Drosophila* the adult cephalic and thoracic structures develop from imaginal discs, small groups of cells set aside during embryogenesis that proliferate during larval development and undergo morphogenesis during the late larval and pupal stages. Recently, Dr. Jim Williams showed that the *vestigial* gene plays a key role in wing and haltere formation. This nuclear protein is first expressed very early during the development of the wing and haltere imaginal discs and is expressed at high levels in the late third instar imaginal cells that will give rise to wing and haltere structures, but not expressed in those disc cells that give rise to structural components of the thorax. In the absence of *vestigial*, the fly completely lacks wings and halteres. These results suggest that the *vestigial* gene product acts to distinguish cells that will become

part of the flight appendages from those that will be part of the thorax proper.

To identify other genes involved in wing formation that may regulate the remarkable pattern of *vestigial* expression, Dr. Williams and Dr. Stephen Paddock have analyzed the developmental roles of and interactions between *vestigial* and three other genes that are required for wing formation. The dynamic patterns of *wingless*, *apterous*, and *vestigial* expression reflect the progressive division of the growing imaginal disc into several distinct subregions that will give rise to different parts of the wing and notum. The *wingless* gene—the earliest acting member of the genetic hierarchy guiding wing development—is required to set up the distinct patterns of *apterous* and *vestigial* expression and that of the gene *scalloped*, which appears to act in concert with *vestigial* to promote the differentiation of wing tissue.

Evolution of Developmental Mechanisms

It is not known whether the developmental mechanisms found in *Drosophila* are conserved throughout different taxa. Julie Gates has shown that within the order Diptera (two-winged insects), the above-mentioned *hairy*, *achaete/scute*, and *vestigial* proteins appear to exhibit very similar, if not identical, patterns of expression throughout the development of nearly a dozen species that are up to 80 million years diverged from *D. melanogaster*. To compare segmentation, neurogenesis, and wing formation in much more divergent insects, Dr. Lisa Nagy, Jim Skeath, and Dr. Williams have isolated segmentation, proneural, and "pro-wing" genes from a number of species representing key insect orders. The expression of these genes in different species may reveal fundamental differences in how insects are organized. For example, there is some doubt as to whether pair-rule genes, so critical for the *Drosophila* mode of development (and highly conserved within these flies), function as segmentation genes in other insects. The availability of molecular probes for key regulatory genes may shed light on the evolutionary history of insects and the genetic basis of morphological diversification.

Advances in Imaging Technology

Research in many cell and developmental biology laboratories is being aided by more powerful techniques for imaging large and complex biological specimens. Dr. Paddock has refined and developed new techniques for visualizing gene expression by immunofluorescence labeling and laser scanning confocal microscopy. The relative spatial and temporal patterns of up to three different proteins can

now be displayed simultaneously and with remarkable clarity with techniques developed by Dr. Paddock, Jim Langeland, and Peter DeVries.

Dr. Carroll is also Associate Professor of Molecular Biology, Genetics, and Medical Genetics at the University of Wisconsin–Madison.

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HORMONAL REGULATION OF GENE EXPRESSION

William W. Chin, M.D., *Investigator*

Dr. Chin and his laboratory are defining the molecular mechanisms by which hormones regulate gene expression. Specifically, he has focused on the role of nuclear receptors and auxiliary proteins in thyroid hormone (TH) action. Further knowledge in this area will increase understanding of the mechanisms of action of ligand-regulated transcription factors such as TH receptor (TR) and the role of TR and other factors in TH action in health and, in particular, in syndromes of TH resistance.

Thyroid Hormone Receptors and Related Forms

TH exerts its diverse physiological actions largely by binding to specific high-affinity nuclear receptors. At least three nuclear TRs have been identified and characterized in the rat: TRα1, TRβ1, and TRβ2. The β forms of the receptor are products of alternative promoter choice and RNA splicing of transcripts derived from a gene that, for human TRβ, is located on chromosome 3; TRα1 is encoded by a separate gene on chromosome 17. Alternative splicing of the TRα gene also generates a non-TH-binding protein, c-erbAα2, which differs from TRα1 only at the carboxyl terminus. Although it does not bind TH, c-erbAα2 has been shown in transient transfection experiments to block the effect of co-transfected TR in facilitating TH regulation of a third co-transfected reporter gene. All forms of TR, along with c-crbAα2, show significant homology to other members of the steroid thyroid hormone receptor superfamily, in particular to the vitamin D and retinoic acid receptors. The three forms of TR have different tissue distributions: TRβ1 is widely expressed in many different tissues; TRβ2 is detected in the pituitary and limited regions of the central nervous system; TRα1, which is expressed in most TH-responsive tissues, is most abundant in skeletal muscle and brown fat.

Structure-Function of Thyroid Hormone Receptors

The ligand-binding domain of TR contains poorly characterized subdomains involved with ligand binding, transactivation, and protein-protein interactions. The region between residues 288–331 of rat TRα1 was analyzed by modeling and site-directed mutagenesis. These results suggest that part of this sequence adopts an amphipathic α-helical conformation. The integrity of the putative helix is important for TH binding but not necessarily for heterodimerization with nuclear factor(s). Mutants defective for both activities were found clustered in a region overlapping the carboxyl-terminal portion of the helix and further downstream. The sequence conservation of this particular region among the entire superfamily suggests a similar role in dimerization in other receptors.

TR Isoform-Specific Antibodies

To examine the expression of these TRs at the protein level, Dr. Chin and his colleagues prepared isoform-specific polyclonal antibodies against the rat TRs and c-erbAα2. Anti-TRβ1 and anti-TRα-common antibodies immunoprecipitate TRβ1 or TRα1, respectively, in transfected COS-7 cells and immunostain almost all of the rat and human anterior pituitary cells, suggesting that TRα1, TRβ1, TRβ2, and c-erbAα2 are most likely expressed in all anterior pituitary cell types in rats and humans. The staining by the anti-TRβ antibodies was primarily

nuclear, as expected; however, surprisingly both cytoplasmic and nuclear staining were observed for the anti-α antibodies, suggesting that the intracellular distribution of c-erbAα2 and/or TRα1 may be different than the distribution of TRβs in the pituitary. The staining of rat pituitary glands by the anti-TRβ2 antibodies demonstrates for the first time that TRβ2 is expressed as a protein in pituitary cells. Furthermore, the staining of human pituitary glands by the anti-TRβ2 antibodies suggests that there is a human homologue of the rat pituitary-specific TRβ2 that shares similar epitopes with the rat TRβ2. Thus isoform-specific antibodies against TRs that can recognize *in vitro* translated, transiently transfected, and *in situ* rat and human pituitary TRs have been prepared.

Homo- and Heterodimers in Thyroid Hormone Action

Dr. Chin and others previously showed that TR binding to thyroid hormone response elements (TREs) can be enhanced by interaction with a nuclear protein, TR auxiliary protein (TRAP). He has studied the binding of TRα1 and TRβ1 to several TREs: the chick lysozyme TRE (F2), which is positively regulated by T$_3$ (3,5,3'-triiodothyronine); rabbit β-myosin heavy-chain TRE (β-MHC), which is negatively regulated by T$_3$; and an idealized inverted palindrome, TRElap. The formation of homodimers, TRα-TRβ dimers, and TR-TRAP heterodimers when receptor is bound to these DNA sequences was demonstrated. Surprisingly, TH decreased in a dose-dependent manner TRα1 and TRβ1 homodimer binding to these TREs as well as TRα-TRβ dimer binding to F2 but did not affect TR-TRAP heterodimer binding to TREs. Recently others have demonstrated that retinoid X receptor (RXR) may be a TRAP and that, in addition to TRAP and RXR, TR heterodimerizes with retinoic acid receptor (RAR) on natural and synthetic hormone response elements. The effect of TH on TR-RAR and TR-RXR heterodimer binding to DNA was also examined. TR formed heterodimers with RAR and RXR on a retinoic acid response element and two TREs. Surprisingly, TH, but not retinoic acid (RA), decreased TR-RAR heterodimer binding to DNA. In contrast, neither TH, all-*trans* RA, or 9-*cis* RA affected TR-RXR binding to DNA. These findings suggest that the TR-RXR heterodimer is also, along with TR-TRAP, a stable receptor complex that remains bound to response elements in the presence of ligand and therefore may be a receptor complex involved in TH-regulated transcription.

Mechanism of Defective TH Signaling in the Syndrome of Generalized Resistance to TH

Generalized resistance to TH (GRTH) is a syndrome of hyposensitivity to T$_3$ (TH) that often displays autosomal dominant inheritance. The genetic defect commonly lies in the ligand-binding domain of one of the TRβ alleles. Since there are two major TR isoforms, TRα and TRβ, it is not known how the mutant receptor mediates a dominant negative effect. The electrophoretic mobility shift assay was employed to compare the effect of TH on the DNA binding of mutant TRβ1 from a kindred with GRTH (Mf-1) with TRβ Mf-1 bound better as a homodimer than TRβ but dissociated from DNA only at high TH concentrations. Both receptors heterodimerized with nuclear auxiliary proteins. They also dimerized with TRα and with each other. Surprisingly, TH disrupted the DNA binding of the Mf-1–TR isoform dimers. Thus mechanisms for the dominant negative effect by mutant TRs likely involve either increased binding to TREs by mutant homodimers that cannot bind TH (hence cannot dissociate from DNA) and/or the formation of inactive mutant TR–nuclear protein heterodimers.

Dr. Chin is also Associate Professor of Medicine at Harvard Medical School, Senior Physician at Brigham and Women's Hospital, and Clinical Associate in Medicine at Massachusetts General Hospital, Boston.

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TRANSCRIPTIONAL REGULATION OF CELLULAR DIFFERENTIATION

GERALD R. CRABTREE, M.D., *Associate Investigator*

Tracing a Signal Transmission Pathway from Cell Membrane to Nucleus

The immune response begins when antigen is presented to a T cell eliciting a characteristic series of cellular changes referred to as T cell activation. Included among these changes are the production of cytokines that control cell fate decisions and differentiation and proliferation among precursors of B cells, granulocytes, and macrophages. The production of these cytokines by activated T cells is largely responsible for coordinating the actions of cells involved in the immune response, and their lack probably explains the severe effects of removing T cell function, as in AIDS (acquired immune deficiency syndrome) or with drugs such as cyclosporin A. Efforts in Dr. Crabtree's laboratory have led to an understanding of several of the steps involved in transmitting signals from the antigen receptor to the early activation genes responsible for the coordination of the immune response.

The Calcium-activated Phosphatase Calcineurin Relays Signals from the Cell Membrane to the Nucleus

The drugs cyclosporin A and FK-506 block the transfer of signals from the cell membrane of T cells to the nucleus by acting at a previously unknown step. Past studies done in the Crabtree laboratory, as well as those of Drs. Walter Neubert in Germany and Mike Hall in Switzerland, led to the formulation of the hypothesis that these drugs produce their effects by forming an inhibitory complex with their binding protein cyclophilin. This led to the search for

molecules that interact with this inhibitory complex. Proteins of 57, 17, and 14 kDa were found by Jeff Friedman (Dr. Irving L. Weissman's laboratory, HHMI, Stanford University) that tightly and specifically bound the inhibitory complex *in vitro* formed between cyclosporin A and its receptor cyclophilin. Dr. Jun Lui in Dr. Stuart Schreiber's laboratory (Harvard University) was able to identify these proteins as calcineurin A, calcineurin B, and calmodulin. These proteins were known to act together to form a highly specific phosphatase. Direct biological evidence for the role of these proteins was obtained by Dr. Neil Clipstone in the Crabtree laboratory. Dr. Clipstone found that overexpression of calcineurin A and B made cells resistant to the drugs and, more significantly, rendered cells far more sensitive to activation than are normal cells. These studies both established calcineurin as the target of action of the drugs cyclosporin A and FK-506 and implicated calcineurin as a key signaling enzyme in T cell activation.

The Regulated Nuclear Entry of NFATc
Is an Element of the Signal
Transfer Pathway

The question that arises from the studies above is that of the relevant substrate for calcineurin. Dr. Mike Flanagan in the Crabtree laboratory began to explore this question by developing an *in vitro* transcription system that would faithfully mimic the complex requirements for T cell activation. He was successful in doing this in every respect except that the extracts of T cells treated with cyclosporin A did not show inhibition of *in vitro* transcription, even though the cells from which they were prepared demonstrated a complete block in transcription dependent on either the entire interleukin-2 (IL-2) enhancer or a trimer of the NFAT (nuclear factor of activated T cells) site. Apparently something was occurring *in vivo* that could not be detected *in vitro*. Dr. Flanagan found that the drug cyclosporin A specifically blocked the entry of a component of NFAT into the nucleus and thereby prevented IL-2 gene activation. Since nuclear and cytoplasmic components are mixed in the *in vitro* transcription extracts, this explained the failure to see an effect of cyclosporin on *in vitro* transcription. These studies indicate that calcineurin is either directly or indirectly required for the entry of NFATc (cytoplasmic subunit of NFAT) into the nucleus. Dr. Jeff Northrop and Dr. Flanagan are presently purifying the cytosolic component of NFATc to determine if it is a direct substrate for the phosphatase activity of calcineurin.

Delineation of a Transcriptional Cascade
Involved in Cell Type Specification

In studies funded in part by the National Institutes of Health, Calvin Kuo, a graduate student in Dr. Crabtree's laboratory, has explored the question of how homeodomain-containing proteins function to specify cell type. Dr. Crabtree and his colleagues reasoned that these proteins must be responsive to signals from their neighbors and hence began looking at the events that regulate the expression of a homeodomain protein that was initially identified in the Crabtree laboratory. This protein, HNF-1 (hepatocyte nuclear factor-1), controls expression of a large group of genes expressed in endodermally derived tissues. HNF-1 is the only known homeodomain protein that can form both homo- and heterodimers in solution and hence has the potential to formulate developmental codes based on regulated dimerization. Calvin Kuo found that HNF-1 was controlled by the transcription factor HNF-4 discovered by Dr. James Darnell and his colleagues (Rockefeller University). HNF-4 is highly related to the predicted product of the *Forkhead* gene in *Drosophila*, mutations in which lead to abnormal head and endodermal development. HNF-4 induces HNF-1 expression and differentiation, as determined by expression of proteins (such as albumin) characteristic of terminally differentiated cells when HNF-4 is transfected into undifferentiated cells. These studies establish a cascade governing terminal differentiation that the laboratory has termed the HNF-1 → HNF-4 hierarchy.

Definition of a Transregulator
of Homeodomain Protein Function

Studies in *Drosophila* involving the exchange of domains within homeodomains have led to the conclusion that the products of certain genes must act to modulate the function of the homeodomain. In an analogous experiment, Dr. Crabtree and his colleagues found that HNF-1 was inactive when transfected into some cell lines but in others could convey a high degree of transcriptional activation to its target promoters. These results implied that certain proteins were necessary for the function of HNF-1. Dirk Mendel in the laboratory set out to find them by looking for proteins that directly associate with the HNF-1. By altering the standard method of oligonucleotide affinity chromatography, he was able to co-

purify an 11-kDa protein in a one-to-one molar ratio with HNF-1. This protein was sequenced and found to be a "pioneer"; i.e., it was not related to other proteins. Overexpression of the protein activated HNF-1–dependent transcription by >100-fold. Since the protein is a cofactor for HNF-1, it was named DCoH. Further studies have indicated that this protein functions by enhancing the stability of HNF-1 dimers and is present within the complex at a ratio of 1:1, indicating that normally HNF-1 is part of a heterotetrameric complex. The regulatory potential for such a heterotetrameric complex could be quite large if there is a family of proteins that participates in this complex. Hence Dr. Crabtree and his colleagues are searching for additional members of this complex.

Dr. Crabtree is also Associate Professor of Pathology at Stanford University School of Medicine.

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THE MECHANISM OF A BACTERIAL TRANSPOSITION REACTION

Nancy L. Craig, Ph.D., *Associate Investigator*

Transposition is a recombination reaction in which a mobile element translocates between nonhomologous positions in DNA. Dr. Craig's research is focused on understanding the transposition mechanism of the bacterial transposon Tn7. A Tn7 transposition is being defined in molecular terms through dissection of the macromolecular interactions between the proteins mediating this reaction and the DNA substrates on which they act.

The Tn7 transposition machinery is elaborate. Tn7 encodes five transposition proteins, TnsA, TnsB, TnsC, TnsD, and TnsE; overlapping subsets of these proteins mediate Tn7 insertion into alternative types of target sites. The Tn7 termini contain complex arrays of DNA sites that are the DNA substrates of this protein machinery.

A subset of the Tns proteins—TnsA + TnsB + TnsC—forms the "core" transposition machinery. These proteins execute the DNA strand cleavages that separate Tn7 from the donor backbone and the strand transfer reactions that subsequently join the transposon ends to the target. However, these proteins alone are inactive; TnsD and TnsE are alternative activators of the TnsA + TnsB + TnsC core machinery. TnsD activates TnsA + TnsB + TnsC to promote high-frequency Tn7 insertion in a specific site in the *Escherichia coli* chromosome called *attTn7*, whereas TnsE provokes these proteins to

mediate low-frequency insertion into many different random sites.

Transposition involves specific recognition of the transposon ends and the target DNAs, juxtaposition of these substrates, strand cleavage to excise the transposon and expose its termini, and strand transfer to join the 3′OH transposon ends to the target. These breakage and joining reactions appear to occur in an elaborate protein-DNA complex containing the substrate DNAs and the Tns proteins. Roles for some of the proteins have been established. TnsB plays a central role in transposon recognition, because it is a specific DNA-binding protein that interacts with both Tn7 ends. A non–sequence-specific DNA-binding protein whose interaction with DNA requires ATP, TnsC plays a key role in target recognition. The role of TnsA remains to be established clearly; one attractive hypothesis is that TnsA mediates interaction(s) between TnsB, bound to the transposon ends, and TnsC, bound to the target DNA. How do TnsD and TnsE activate the core machinery? TnsD is a sequence-specific DNA-binding protein that binds specifically to *attTn7* and promotes the interaction of TnsC with *attTn7*, thereby bringing the core machinery and transposon ends to the insertion point. Perhaps TnsE is a low-specificity DNA-binding protein that positions the core machinery at many places on target DNA.

Dissecting the Mechanism of DNA Strand Breakage and Joining

The fundamental reactions in transposition are DNA strand cleavage to expose the transposon ends and strand transfer to join the ends to the target DNA. The current major focus of the research in Dr. Craig's laboratory is to determine in chemical detail how these reactions occur and to define the active sites that promote them.

It is suspected that these activities are provided by TnsB, the protein that interacts specifically with the transposon ends. This hypothesis arose because Dr. Craig and her colleagues were able to align part of the TnsB amino acid sequence with highly conserved sequences of several retroviral and retrotransposon integrases; others have hypothesized that this conserved motif plays a key role in DNA strand cleavage and transfer by providing an essential metal cofactor-binding site.

Several experimental approaches are being used to determine if TnsB does indeed contain the active site for transposition. One strategy will be to change highly conserved amino acids in TnsB by site-directed mutagenesis. A more powerful approach is to isolate gain-of-function TnsB mutants in which these usually repressed activities are artificially activated. Dr. Craig and her colleagues are seeking such mutants in the presence of various combinations of the other Tns proteins because they suspect that multiple protein-protein interactions and protein-DNA interactions modulate the activity of TnsB.

Another strategy is to use alternative or modified DNA substrates. Using work with retroviral integrases as a base, Dr. Craig and her colleagues are developing a novel Tn7 substrate in which a transposon end is already joined to one strand of the target DNA. Others have shown that the exposed target end can participate in novel transposase-dependent reactions of reduced stringency, providing a powerful tool for examining low-level uncoupled reactions and the strand transfer chemistry. This novel strand transfer reaction with the Tns proteins has been observed in this laboratory, and the requirements and characteristics of this unusual reaction are being examined.

Dr. Craig and her colleagues also wish to examine in detail the elaborate protein-DNA complex in which transposition occurs and, in particular, to define the specific interactions between the various Tns proteins. They anticipate that affinity chromatography and protein crosslinking will be important tools in this work.

Control of Tn7 Transposition

Tn7 transposition is highly controlled. For example, although TnsA + TnsB + TnsC contains the active sites for transposition, no transposition occurs unless the activators TnsD or TnsE are also present. The interaction of TnsC with the target DNA is a critical control step. For example, changing TnsC's ATP cofactor has a profound effect on transposition; moreover, TnsD appears to activate transposition by promoting the stable interaction of TnsC with *attTn7*. TnsC mutants that activate the core machinery in the absence of TnsD or TnsE have recently been isolated. A number of amino acid changes that provide activated TnsC have been identified. Understanding why these mutant proteins are activated will provide further insight into the control of wild-type TnsC. Another complementary approach is the effort in Dr. Craig's laboratory to reconstitute TnsE-dependent transposition *in vitro* with purified proteins. Being able to manipulate both the high-frequency TnsD pathway and low-frequency TnsE pathway *in vitro* will be useful in dissecting transposition control.

Another feature of Tn7 transposition that reflects the highly controlled nature of this reaction is that Tn7 displays transposition immunity; that is, Tn7

does not insert into target DNAs that already contain a copy of Tn7. Dr. Craig and her colleagues have recently established that Tn7 transposition immunity is active in the *Escherichia coli* chromosome.

The work on the control of Tn7 was supported by a grant from the National Institutes of Health.

Dr. Craig is also Associate Professor of Molecu- *lar Biology and Genetics at the Johns Hopkins University School of Medicine.*

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MECHANISM OF RETROVIRUS INFECTION

JAMES M. CUNNINGHAM, M.D., *Assistant Investigator*

Infection by retroviruses requires successful interaction between viral and host cell proteins. In the appropriate target cells, infection may result in transformation or cell death. Dr. Cunningham and his colleagues are interested in understanding how retroviruses infect cells and in determining the consequences of infection for the host. They have focused on the behavior of a membrane protein that functions as a transporter of cationic amino acids and serves as a receptor for leukemogenic retroviruses (murine leukemia virus, MuLV) in mice.

Role of the Transporter in Infection

Previous work in Dr. Cunningham's laboratory established that the transporter determines infection by binding to the MuLV envelope. Site-directed mutagenesis has identified a transporter domain that provides the site for virus attachment on the extracellular face of the plasma membrane. Binding to the transporter is a prerequisite for fusion of the virus envelope to the host cell membrane, which may occur after delivery of the virus to endosomes. Currently the laboratory is investigating the role of the transporter in these steps by screening for mutant transporter proteins that bind to the virus envelope but do not permit infection. The laboratory is also using crosslinking reagents and antibodies to look for host cell proteins that interact with the virus envelope and matrix proteins in the presence of the transporter.

In the course of the experiments designed to identify mutant transporter proteins, Dr. Cunningham and his co-workers recognized that synthesis of complementary DNA from the viral RNA template begins within virions prior to infection. Previously it had been believed that replication of the MuLV genome by reverse transcriptase was initiated in the cytoplasm of the host cell after infection.

These findings demonstrate the importance of the cleavage of active reverse transcriptase from the virus-encoded gag-pol polyprotein within maturing virions. In addition, they suggest that inhibition of retrovirus replication need not occur within infected cells.

Consequences of Retrovirus Infection for the Host Cell

Dr. Cunningham and his colleagues have been investigating virus interference—the resistance of infected cells to additional infection by MuLVs that use the same receptor. Interference is a consequence of binding of newly synthesized virus envelope to the transporter. Binding of the MuLV envelope protein to the transporter in the endoplasmic reticulum results in a decrease in the delivery of the transporter to the cell surface, increased turnover of the transporter, and an increase in the steady-state level of the transporter-encoding mRNA. Currently the laboratory is testing the hypothesis that the increase in transporter mRNA within infected cells may be a response to depletion of intracellular cationic amino acids, which results from the decrease in functional transporter at the plasma membrane. In addition, altered glycosylation of the transporter in infected cells has been observed, and two asparagine residues within the envelope-binding domain have been identified that are the sites for glycosylation. The modification of N-linked carbohydrate during passage of the transporter through the Golgi may be altered when bound to envelope protein. Subsequent experiments using site-directed mutagenesis have excluded a requirement for glycosylation in transporter-mediated amino acid uptake and MuLV infection. The possibility that an altered carbohydrate structure on the transporter may be important for interference is now being examined.

Previously Dr. Cunningham's laboratory showed that the virus receptor is not expressed in liver. In the past year, they have identified a second transporter of cationic amino acids that is related to the virus receptor and is expressed in hepatocytes. This transporter has a much lower affinity—but greater transport capacity—for cationic amino acids than the MuLV receptor. These properties permit hepatocytes to remove high levels of these amino acids from the portal vein after a large protein meal, but protect the plasma pool of arginine from the intracellular arginase required for urea synthesis within hepatocytes.

A third cationic amino acid transporter arises by alternative splicing of mRNA derived from the gene encoding the liver transporter. Expression of this transporter is induced in macrophages activated by interferon-γ or tumor necrosis factor. These cytokines also decrease the expression of the virus receptor. The inducible transporter may be required to supply macrophages with arginine, the substrate for synthesis of nitric oxide (NO), an important signaling molecule that moves freely from macrophages to adjacent cells and can activate guanylate cyclase, resulting in the production of cGMP. In addition, NO is a highly reactive free radical that is toxic to tumor cells and to intracellular pathogens, including those that cause tuberculosis, schistosomiasis, leishmaniasis, toxoplasmosis, and malaria. In the past year, Dr. Cunningham and his co-workers have cloned a cDNA from activated macrophages that encodes nitric oxide synthase (NOS), the enzyme that catalyzes the synthesis of NO from argi-

nine. Using this clone, they have demonstrated coordinate expression of NOS and the transporter by cytokines. They are currently investigating the consequences of MuLV infection on NO production in macrophages.

Dr. Cunningham is also Assistant Professor of Medicine at Harvard Medical School and Associate Physician at Brigham and Women's Hospital, Boston.

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GENETIC ANALYSIS OF NUCLEOCYTOPLASMIC TRANSPORT

Laura I. Davis, Ph.D., *Assistant Investigator*

Research in Dr. Davis's laboratory is aimed at understanding how macromolecular transport across the nuclear envelope is controlled. The populations of RNA and protein found in the nucleus and cytoplasm are very different. Newly synthesized mRNA, for example, is normally fully processed before moving into the cytoplasm. Similarly, only proteins containing specific signals are imported into the nucleus. Recently it has become clear that some mediators of the signal transduction pathway exert their effect on transcription by modifying cytoplasmic transcription factors so as to allow their entry into

the nucleus. Thus nucleocytoplasmic transport is an important regulatory point in the control of gene expression.

Transport proceeds through large proteinaceous channels called nuclear pore complexes. These channels have an overall diameter of ~120 nm and have been estimated to contain up to 200 different polypeptides. It is likely that some pore proteins function to define the specificity of transport, whereas others carry out the actual translocation step. Among the few known components of the nuclear pore complex is a family of polypeptides,

called the nucleoporins, that were first identified in mammalian cells. *In vitro* assays performed by other laboratories suggest that the nucleoporins play an essential role in mediating protein (and perhaps RNA) transport, but their exact function remains unknown.

Dr. Davis's goal is to combine genetics and biochemistry to elucidate the function of each of the nucleoporins and to identify other components of the transport apparatus. To accomplish this, she uses the budding yeast *Saccharomyces cerevisiae* as an experimental system, because it is amenable to both genetic and biochemical analysis. Dr. Davis previously identified several yeast homologues of the mammalian nucleoporins and cloned the gene encoding one of them (*NUP1*).

Random mutagenesis has been used to identify mutations in *NUP1* that confer temperature-sensitive growth. Immunofluorescence assays were employed to examine the efficiency of protein import as well as mRNA export in one such mutant. This mutant failed to import an inducible nuclear protein upon shifting to the nonpermissive temperature. The mutant also accumulated polyadenylated RNA in the nucleus. These results suggest that *NUP1* is required for both protein import and RNA export. Thus the elucidation of cellular proteins that functionally interact with *NUP1* should provide valuable insights into the mechanisms that govern these two processes.

While most yeast strains require *NUP1* for viability, a strain that carries a naturally occurring suppressor that bypasses this requirement has recently been identified. Deletion of *NUP1* in this strain background causes cells to grow more slowly than normal, suggesting that the bypass is not complete. By analogy to other systems, mutations in genes encoding proteins that functionally interact with *NUP1* might be expected to exacerbate the effect of *nup1* deletion and prevent growth. These "synthetic lethals" can be identified by screening for mutants that can no longer grow in the absence of *NUP1*. An initial screen has identified several such mutants, which remain to be characterized. The same question can be addressed directly by crossing *nup1* deletion strains to known mutants suspected

of functioning in the same pathway. The *RNA1* gene, characterized by Dr. Anita Hopper and her colleagues, encodes a cytosolic protein apparently required for export of RNA. Dr. Davis's group has found that *rna1-1* exhibits synthetic lethality with the suppressed *nup1* deletion. This observation suggests that these two proteins interact functionally and lends credence to the idea that the genetic screen will identify functionally relevant proteins.

NUP1p presumably functions through physical association with other proteins. These may be other pore complex constituents or nuclear or cytoplasmic proteins that interact transiently with the pore complex. Overexpression of NUP1p, which causes cytosolic accumulation of the overexpressed protein, is lethal to cells. One explanation for this is that cytoplasmic NUP1p binds to and conscripts a protein or proteins with which it would normally interact in the nuclear pore complex. The region of NUP1p that mediates this phenotype has been localized to the amino-terminal domain. This domain also correlates with the ability of *NUP1* to function, because deletions and point mutations within this region render the protein nonfunctional. A biochemical approach has been used to identify and purify two nuclear proteins that bind to this domain. To test the relevance of this interaction, nonfunctional amino-terminal mutants are now being tested for their ability to bind these proteins. If a correlation between *in vitro* binding and *in vivo* function can be established, then efforts to clone the genes encoding these proteins will immediately ensue. A complementary genetic approach has also been initiated. Several clones have been isolated that, when expressed in high copy, can rescue the lethality of *NUP1* overexpression. Characterization of these genes is currently under way.

Dr. Davis is also Assistant Professor of Genetics and Cell Biology at Duke University Medical Center.

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SIGNAL TRANSDUCTION BY GROWTH FACTOR RECEPTORS

Roger J. Davis, Ph.D., *Assistant Investigator*

The molecular biology of transmembrane signal transduction by cell surface receptors for polypeptide growth factors is the major focus of Dr. Davis's laboratory. To gain an understanding of the properties of a typical cell surface receptor, the laboratory has initiated a detailed analysis of the structure-function relationships for the transferrin receptor. In addition, further studies are in progress that are designed to establish the molecular basis of epidermal growth factor (EGF) receptor function and the mechanism of mitogenic signaling by this receptor.

Signal Transduction Pathways Activated by the EGF Receptor

One mechanism of signaling by the EGF receptor is the stimulation of the activity of protein kinases located within signal transduction pathways. The molecular characterization of these enzymes is therefore an important goal. Dr. Davis and his colleagues previously demonstrated that the major site of EGF-stimulated phosphorylation of the EGF receptor is located at Thr^{669}. The growth factor–stimulated phosphorylation of the receptor at this site can be accounted for by MAP (mitogen-activated protein) kinases that are activated by phosphorylation on tyrosine and threonine residues.

During the past year, four human isoforms of this kinase family were molecularly cloned in Dr. Davis's laboratory: $p40^{mapk}$, $p41^{mapk}$, $p44^{mapk}$, and $p63^{mapk}$. Immunofluorescence analysis using both confocal and three-dimensional reconstruction techniques have shown that the $p63^{mapk}$ isoform is predominantly located within the cytoplasm and is largely excluded from the nucleus. In contrast, the $p40^{mapk}$, $p41^{mapk}$, and $p44^{mapk}$ isoforms are localized in both the cytoplasmic and nuclear compartments of cells. Significantly, it was found that growth factor treatment caused an increase in the nuclear accumulation of these protein kinases.

The growth factor–stimulated nuclear translocation of MAP kinases suggests that these enzymes may have a significant role in the process of signal transduction between cell surface receptors and the nuclear targets of growth factor action. To identify potential nuclear substrates for the MAP kinases, Dr. Davis and his colleagues examined the substrate specificity of the purified kinases. Substrates identified include the EGF receptor (Thr^{669}), c-Myc (Ser^{62}), and c-Jun (Ser^{246}). Inspection of the pri-

mary sequences surrounding these phosphorylation sites indicates the consensus sequence Pro-Leu-Ser/Thr-Pro. A systematic analysis of the effects of point mutations within this region demonstrated that the consensus sequence for phosphorylation can be generalized as Pro-Xaa_n-Ser/Thr-Pro (where Xaa is an aliphatic or basic amino acid, and $n = 1$ or 2). The identification of this consensus sequence has great value, because it allows the prediction of potential MAP kinase phosphorylation sites, using the sequence of cloned proteins.

Regulation of c-Myc Function by MAP Kinases

Dr. Davis and his colleagues previously identified the major site of growth factor–regulated phosphorylation of c-Myc as Ser^{62}. This phosphorylation site, which is located within the transcriptional activation domain of c-Myc, is a substrate for phosphorylation by MAP kinases. During the past year studies have been performed that are designed to establish the physiological significance of the phosphorylation of c-Myc at the MAP kinase phosphorylation site. A GAL4 fusion protein strategy was employed to examine the effects of phosphorylation on transcriptional activation. It was found that the phosphorylation site is required for high levels of transactivation of gene expression. Thus it is likely that the MAP kinase phosphorylation site (Ser^{62}) represents a physiologically important mechanism of regulation of the transcriptional activity of the c-Myc protein.

Role of Multisite Phosphorylation of the EGF Receptor

The EGF receptor is a 170-kDa transmembrane glycoprotein that is expressed at the cell surface. The receptor is composed of an extracellular domain that binds EGF, a single transmembrane-spanning domain, and a cytoplasmic domain with intrinsic protein-tyrosine kinase activity. Binding of EGF to the extracellular domain of the receptor stimulates the cytoplasmic domain protein-tyrosine kinase activity and results in the autophosphorylation of the receptor and the phosphorylation of exogenous substrates, e.g., phospholipase C-γ and GTPase-activating protein (GAP). This process of transmembrane signaling is regulated by multisite phosphorylation of the receptor at serine and threonine residues.

One regulatory site of phosphorylation, Thr^{654}, is a substrate for protein kinase C. Phosphorylation of the EGF receptor at this site causes an inhibition of the receptor tyrosine kinase activity and accounts for the inhibition of signal transduction caused by phorbol ester. However, this mechanism does not account for the physiologically relevant desensitization of the receptor that occurs after the treatment of cells with EGF. During the past year Dr. Davis's laboratory demonstrated that a serine phosphorylation site located within the carboxyl-terminal domain of the receptor (Ser$^{1046/7}$) is required for EGF-stimulated desensitization of the EGF receptor. Mutation of the receptor at Ser$^{1046/7}$ blocks the homologous desensitization of the receptor tyrosine kinase activity and causes potentiation of signal transduction. Significantly, the *in vitro* phosphorylation of the EGF receptor at Ser$^{1046/7}$ caused an inhibition of the receptor tyrosine kinase activity. These studies demonstrate that there are two independent mechanisms of regulation of EGF receptor function by phosphorylation. Because autocrine and paracrine stimulation of the EGF receptor can contribute to tumor growth, the ability to manipulate EGF receptor function by multisite phosphorylation has implications for the design of novel antitumor drugs.

Disease Potential of the *erbB* Oncogene

Direct evidence for the involvement of the phosphorylation site Ser$^{1046/7}$ in oncogenic transformation was obtained from studies of *erbB*. Retroviruses can cause erythroleukemia after a long latent period by inserting within the c-*erbB* gene. This insertion results in the expression of a truncated EGF receptor with constitutive protein-tyrosine kinase activity. The insertionally activated *erbB* oncogene stimulates the self-renewal of erythrocytic progenitor cells and is exclusively leukemogenic. In contrast, acute transforming viruses that carry the *erbB* oncogene can induce both sarcomas and erythroleukemias. This expanded oncogenic potential of *erbB* can be attributed to structural alterations in the *erbB* protein. Comparison of the deletions present within the *erbB* gene in a series of viruses that exhibit an expanded disease tissue tropism indicates that the deletion of the phosphorylation site Ser$^{1046/7}$ is a common event.

To test the hypothesis that the mutational removal of this phosphorylation site is relevant to the increased disease potential of *erbB*, Dr. Davis and his colleagues constructed recombinant retroviruses with defined mutations. Infection of animals with the recombinant viruses demonstrated that the replacement of Ser$^{1046/7}$ with Ala residues causes the rapid formation of sarcomas. Thus the loss of the negative regulatory phosphorylation site Ser$^{1046/7}$ causes an expansion of the tissue specificity of tumor formation by the *erbB* oncogene. Together these data establish an important functional role for the Ser$^{1046/7}$ phosphorylation site.

Function of the Transferrin Receptor

The major mechanism that accounts for the accumulation of iron by cells is the receptor-mediated internalization of a serum iron-binding protein, transferrin. The ubiquitous expression of the transferrin receptor by proliferating cells reflects the requirement of iron for mitogenesis. An understanding of the molecular mechanisms that account for the function of the transferrin receptor is a goal of this research. The disruption of the transferrin receptor gene has been achieved in Dr. Davis's laboratory in tissue culture cells. These cells provide a suitable model system for the expression of mutant forms of the transferrin receptor. During the past year the effects of point mutations and deletions within the cytoplasmic tail of the transferrin receptor have been systematically analyzed. The results demonstrated that only a small region of the receptor cytoplasmic tail is required for endocytosis. Studies in progress are designed to characterize cellular proteins that interact with the internalization signal sequence present within the tail of the transferrin receptor. This project was supported by a grant from the National Institute of General Medical Sciences, National Institutes of Health.

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RIBONUCLEOPROTEIN COMPLEXES OF HETEROGENEOUS NUCLEAR RNA, NUCLEAR STRUCTURES, AND MESSENGER RNA FORMATION

GIDEON DREYFUSS, PH.D., *Investigator*

Messenger RNAs (mRNAs) are formed in the nuclei of eukaryotic cells by extensive post-transcriptional processing of primary transcripts of protein-coding genes. These transcripts, called heterogeneous nuclear RNAs (hnRNAs) or pre-mRNAs, are produced by RNA polymerase II. From the time hnRNAs emerge from the transcription complex, and throughout the time they are in the nucleus, they are associated with proteins. The collective term for the proteins that bind hnRNAs and that are not stable components of other classes of RNP complexes, such as snRNPs, is hnRNPs. The full range of functions and the mechanism of action of hnRNPs are not known. It can be anticipated, however, that hnRNPs influence the structure of hnRNAs and facilitate or hinder the interaction of hnRNA sequences with other components that are needed for pre-mRNA processing. The hnRNPs may also play important roles in the interaction of hnRNA with other nuclear structures, in nucleocytoplasmic transport of mRNA, and in other cellular processes. They are also major nuclear structures. In addtion, what has been learned from the study of hnRNPs turned out to be extremely instructive for other RNA-binding proteins, including those that control developmentally important pathways, snRNPs and mRNA-binding proteins (mRNPs).

hnRNP Complexes

Photochemical RNA-protein crosslinking in intact cells and affinity chromatography methods have been used to identify and purify the major hnRNPs and mRNPs of vertebrate cells, and monoclonal antibodies to many of them have been produced. These antibodies were used to develop an immunopurification procedure for hnRNP complexes and to begin the characterization of these proteins.

The most detailed picture of the protein composition of hnRNP complexes is presently available for human cells. Considerable information is also becoming available for invertebrates, particularly *Drosophila melanogaster*. Human hnRNP complexes contain large hnRNA (≥ 10 kb) and at least 20 major proteins, designated A–U, in the range of 34–120 kDa. The arrangement of the proteins on specific hnRNAs, which is probably important in determining the structure of the hnRNA, is one of the areas on which much work is focused. Several hnRNPs were found to have RNA-binding specificities. Some of the specificities of the major hnRNPs are for sequences that are important for pre-mRNA processing and polyadenylation, and these binding preferences are probably directly related to roles for these proteins in mRNA formation. Dr. Dreyfuss's laboratory previously described the isolation and sequenc-

ing of cDNAs for the hnRNP A2, B1, C1, C2, I, K, L, and U proteins. Recently, cDNAs of the hnRNP F, G, H, and M proteins have been isolated. The hn-RNP complexes of *D. melanogaster* have also been isolated, and their major proteins have been sequenced.

Structure of hnRNPs

Sequence analysis, mutagenesis, and binding studies of hnRNPs delineated several motifs for RNA binding and protein-protein interaction. Most of the hnRNPs (as well as many mRNA-, pre-rRNA– and snRNA-binding proteins) belong to a large family of RNA-binding proteins that contain one or several highly conserved 90– to 100–amino acid RNP motifs that define an RNA-binding domain (RBD). Dr. Dreyfuss and his colleagues (in collaboration with Drs. Michael Wittekind, Robert A. Beckman, and Luciano Mueller of Bristol-Myers Squibb) have determined the structure of the 93–amino acid RBD of the pre-mRNA–binding hnRNP C protein in solution and mapped candidate amino acids involved in RNA binding by multidimensional nuclear magnetic resonance (NMR). This RBD has a compact folded structure ($\beta\alpha\beta$-$\beta\alpha\beta$) comprising a four-stranded anti-parallel β sheet, two α helices, and relatively unstructured amino- and carboxyl-terminal regions. The RNP1 and RNP2 consensus sequences—which are the most highly conserved peptides of this RNP family—are juxtaposed on the adjacent central β strands ($\beta 3$ and $\beta 1$) of the β sheet and exposed on the surface of the domain. *In vitro* random-sequence selection methods were used to identify specific RNA ligands for this RBD. These experiments demonstrated that the hnRNP C proteins bind to oligo-uridine–containing RNA.

The interaction of the hnRNP C protein RBD with $r(U)_8$ was studied in NMR experiments. These studies revealed that residues in the β-sheet region and in the amino- and carboxyl-terminal regions of the RBD are significantly affected by the formation of the hnRNP C RBD:$r(U)_8$ complex. In contrast, the residues of the well-conserved α helices, with one exception, were not affected. Mutagenesis is being used to identify independently residues within the RBD that are critical for its interactions with RNA. These analyses indicate that a large number of amino acids on the β sheet and the contiguous amino- and carboxyl-terminal regions of the RBD provide an exposed "platform" for extensive interactions with the RNA. RNA bound to this platform should be accessible to other (e.g., splicing) factors, rather than buried in a binding pocket.

Localization and Transport of pre-mRNA–binding Proteins

Immunofluorescence microscopy with most of the hnRNP antibodies shows general nucleoplasmic localization for these proteins with little or no staining in the nucleoli and in the cytoplasm. Much of this signal results from staining of nascent transcripts; experiments on *Drosophila* polytene chromosomes allow observation of the association of individual hnRNPs and snRNPs with specific pre-mRNAs. The nuclear staining was interpreted to indicate that hnRNPs are restricted to the nucleus of interphase cells, with the conclusion that the functions of hnRNPs concern strictly nuclear processes.

However, Dr. Dreyfuss's laboratory recently found that this is not always the case and that some of the hnRNPs (such as those of the A and B groups) shuttle between the nucleus and the cytoplasm. In contrast, other hnRNPs (such as the C and U proteins) appear to be confined to the nucleus. This suggests that the shuttling hnRNPs may also have functions in the cytoplasm, that the hnRNP complexes are dynamic, and that a role for these proteins in nucleocytoplasmic transport of RNA must be considered. All of the hnRNPs must be imported into the nucleus, and some of them, the shuttling proteins, must also be exported to the cytoplasm. A role for polymerase II transcription in the localization of these proteins has also recently emerged. The signals in the hnRNPs that mediate their transport and localization are being investigated.

Dr. Dreyfuss is also Professor of Biochemistry and Biophysics at the University of Pennsylvania School of Medicine.

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PROTEIN SORTING IN THE YEAST SECRETORY PATHWAY

Scott D. Emr, Ph.D., *Associate Investigator*

Dr. Emr's laboratory is using the yeast *Saccharomyces cerevisiae* as a model genetic system to study protein sorting to the lysosome-like vacuole. The laboratory previously designed a gene fusion-based selection scheme that enabled them to isolate more than 600 yeast mutants that exhibit severe defects in vacuolar protein sorting. The recessive mutations in these mutants define more than 33 complementation groups that exhibit defects in vacuolar protein sorting (*vps* mutants). Extensive genetic, biochemical, and morphological characterization of the *vps* mutants indicates that many of the *VPS* genes encode components of the protein-sorting machinery responsible for either the selective recognition and packaging of vacuolar hydrolases into carrier vesicles or the transport and fusion of such vesicles with the correct target membrane. The isolation and characterization of the genes affected in these mutants is providing new insights into the molecular mechanisms that control protein traffic in the eukaryotic secretory pathway.

Characterization of *VPS* Genes Required for Vacuolar Protein Sorting

Dr. Emr's laboratory has cloned and sequenced eight *VPS* genes. Comparison of the *VPS* gene sequences with other known genes has revealed several informative structural and functional similarities. Much of the laboratory's recent efforts have been directed toward the characterization of *VPS15* and *VPS34*, two genes of particular interest. The sequence of the *VPS15* gene predicts a protein with the following features: a consensus site for amino-terminal myristoylation, a region that shares significant sequence similarity with the family of Ser/Thr protein kinases, and a region of homology with the

regulatory subunit of protein phosphatase 2A (PP2A). The sequence similarity the Vps15 protein (Vps15p) shares with Ser/Thr protein kinases and the PP2A regulatory subunit raises the possibility that protein phosphorylation/dephosphorylation reactions may play an important role in the regulation of protein-sorting events. Mutagenesis of several highly conserved amino acid residues in the putative kinase domain of the Vps15p inactivated the complementing activity of the *VPS15* gene. The mutant cells exhibit extreme vacuolar protein-sorting defects. Vacuolar hydrolases like carboxypeptidase Y (CPY) are missorted and secreted by these mutants.

Vps15p is a phosphoprotein, and mutations in its kinase domain eliminate its phosphorylation. These data suggest that the Vps15p is an active kinase and that this kinase activity is required during some step in vacuolar protein sorting. Protein phosphorylation may act as a "molecular switch" in this protein-sorting pathway by actively diverting vacuolar hydrolases away from the default secretion path and toward the vacuole. Several points in the pathway, such as the budding and transport of carrier vesicles or the recognition and fusion of these vesicles with the vacuole, may need to be regulated precisely. A block in any one step could lead to missorting and secretion of vacuolar hydrolases.

Short carboxyl-terminal truncations of Vps15p (removing as little as 30 amino acids) result in a temperature-conditional defect in the delivery of CPY to the vacuole. CPY delivery is essentially wild-type at the permissive temperature but almost completely blocked immediately after shifting cells to the restrictive temperature. Upon imposition of the temperature block, the mutant cells accumulate

newly synthesized p2 CPY within a saturable intracellular compartment that is distinct from the vacuole. This compartment does not appear to represent an aberrant, dead-end structure, because the accumulated p2 CPY can be efficiently processed to its mature form following a shift back to the permissive temperature. The rapid and efficient reversal of the block indicates that CPY is present within a normal intermediate of the vacuolar protein transport pathway. Dr. Emr's laboratory is presently attempting to purify this compartment.

The VPS15 and VPS34 Gene Products Form a Protein Complex

The substrates and activators of the novel protein kinase Vps15p must be identified to determine its precise role in vacuolar protein sorting. A search for multicopy plasmid suppressors of specific vps15 mutations revealed that vps15 kinase domain mutations are suppressed by the overproduction of Vps34p but not by the overproduction of other VPS gene products. In contrast, the vacuolar protein-sorting defects of strains in which the VPS15 gene has been deleted are not suppressed by the overproduction of Vps34p, demonstrating that the overproduction of Vps34p cannot bypass the requirement for Vps15p in vacuolar protein sorting.

These genetic data indicate that Vps15p and Vps34p functionally interact in yeast cells, probably at a common step in the vacuolar protein-sorting process. Native immunoprecipitation and chemical crosslinking experiments have demonstrated that the proteins also physically interact and are coprecipitated by antisera specific for either of the individual proteins. The combined biochemical and genetic data therefore demonstrate that Vps15p and Vps34p act together within a hetero-oligomeric protein complex to facilitate yeast vacuolar protein delivery.

VPS34 Encodes a Lipid Kinase

Recent data have provided some exciting new insights into the role of the Vps15/Vps34 protein complex in the protein-sorting reaction. Vps34p shares significant sequence similarity with the catalytic subunit of mammalian phosphatidylinositol 3-kinase (PI3-kinase). PI3-kinase phosphorylates the membrane lipid phosphatidylinositol (PI) and other more highly phosphorylated PI derivatives (PI-P and PI-P$_2$) at the D-3 position of the inositol ring. In mammalian cells, PI3-kinase associates with many signal-transducing receptor tyrosine kinases (e.g., the platelet-derived growth factor [PDGF], in-

sulin, and colony-stimulating factor-1 [CSF-1] receptors) and is postulated to be involved in the generation of key second messenger molecules important for regulating cell growth and proliferation.

PI3-kinase activity is readily detected in wild-type yeast cell extracts, but extracts of yeast strains deleted for the VPS34 gene exhibit undetectable levels of PI3-kinase activity. In addition, strains overproducing Vps34p show elevated levels of PI3-kinase activity. These data indicate that VPS34 encodes a PI3-kinase activity in yeast and suggest that PI3-kinase activity is involved in regulating intracellular protein sorting. Vps34p-mediated phosphorylation of membrane PI could serve as a signal that triggers the specific interaction, or stabilization, of other proteins required for transport to the vacuole, such as vesicle coat proteins.

One possibility presently being tested is that the Vps15p kinase directly regulates the lipid kinase activity of Vps34p through a specific protein phosphorylation reaction. Vps15p kinase activity may itself be regulated by direct interaction with particular transmembrane receptors, such as a CPY-specific receptor. In such a model, Vps15p and Vps34p effectively act as components of a signal transduction complex that transduces the signal received by the membrane receptors into a second messenger (PI3-phosphate) that could trigger the action of as yet unknown effector proteins that direct protein sorting to the vacuole.

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BIOCHEMISTRY AND PHYSIOLOGY OF THE PROTEIN C ANTICOAGULANT PATHWAY

CHARLES T. ESMON, PH.D., *Investigator*

During blood coagulation, prothrombin must be converted to the serine protease thrombin. Thrombin not only clots blood but also is a major regulatory protein in this process, generating both amplifying and inhibiting signals. How thrombin selects its substrate, and hence whether it functions as a clot-promoting or -inhibiting enzyme, is the major focus of Dr. Esmon's laboratory.

Thrombin functions as an anticoagulant by activating the anticoagulant zymogen protein C. Activation occurs when thrombin complexes with thrombomodulin, an endothelial cell receptor. Compared with the other complexes responsible for activation of coagulation zymogens, both the enzyme thrombin and the regulatory protein thrombomodulin have much simpler structures, suggesting that detailed analysis of this system might serve as a model for the others. Three recent advances are of particular utility: 1) small (10- to 15-kDa) thrombomodulin fragments, corresponding to two (GF5–6) or three (GF4–6) epidermal growth factor (EGF)-like repeats from the intact thrombomodulin molecule, can be formed that bind thrombin and either do or do not accelerate protein C activation; 2) hirudin, a specific thrombin inhibitor from leeches, interacts with thrombin through two separate domains, both of which inhibit thrombin's activity; and 3) the crystal structure of thrombin has recently been determined with and without hirudin bound to the enzyme. Thrombin's structure reveals that it differs from most proteases by having an extended groove that runs almost around the enzyme. This groove is occupied by the carboxyl-terminal portion of hirudin, referred to as hirugen. This groove contains many basic residues and is referred to as the anion-binding exosite. Competition experiments revealed that hirugen and GF5–6 share overlapping binding sites on thrombin and that both fragments elicit comparable conformational changes and alterations in thrombin specificity. When GF4–6 binds thrombin, additional conformational changes occur that result in altered specificity toward small substrates as well as protein C. These results indicate that thrombomodulin interacts with two distinct sites on thrombin that function in concert to alter thrombin allosterically and change its specificity to accommodate the activation site in protein C.

Allosteric changes in specificity also appear to be involved in thrombin-dependent cell activation. Cellular activation involves cleavage of a thrombin receptor, as recently demonstrated by Dr. Shaun Coughlin's group (University of California, San Francisco). Analysis of the structure-function properties of the receptor revealed that a peptide from the receptor binds to thrombin in the anion-binding exosite and elicits a conformational change in thrombin comparable to that produced by GF5–6. Consistent with this concept, thrombin mutants lacking the active-site serine still bind either thrombomodulin or the thrombin receptor and block both the formation of anticoagulant activity and cell activation. These results provide a molecular explanation for how thrombomodulin blocks cell activation by thrombin.

Direct analyses of the assembly of the protein C activation complex were performed in the ultracentrifuge. The ternary complex between protein C, thrombin, and thrombomodulin could be detected and demonstrated a Ca^{2+} dependence consistent with the activation kinetics. Surprisingly, activated protein C bound to the activation complex with the same affinity as the substrate protein C. These observations were confirmed and extended by demonstrating that activated protein C behaves as a competitive inhibitor of protein C activation, with a K_i approximately equivalent to the K_m for protein C. Current work is focused on the possibility that this interaction of activated protein C with thrombomodulin alters the function of activated protein C.

During prothrombin activation, two proteolytic cleavages in the zymogen are required for activation. Only after both cleavages occur does thrombin activate protein C or cells effectively. Direct binding studies were initiated to determine when the anion-binding exosite is formed during prothrombin activation. Surprisingly, both of the potential activation intermediates possess the anion-binding exosite. Thus, although this deep binding pocket is formed in the intermediates, further structural changes are required for productive interaction with cells or thrombomodulin.

Factor V, one of the regulatory proteins in coagulation, is also a thrombin substrate. Relatively little is known about why thrombin interacts with factor V with high affinity. As one approach to investigating this question further, Dr. Enriqueta Guinto in Dr. Esmon's laboratory completed the analysis of the bovine factor V sequence. Because of relative abundance and stability, most of the information currently available about the function of factor V is

based on analysis of the bovine molecule. This study provides a structural basis to understand these functional studies better.

The entire process of blood coagulation requires Ca^{2+}, which is involved not only in interaction of the clotting proteins with cell surfaces but also in protein-protein interactions. In most of the coagulation complexes, understanding whether the Ca^{2+} effect is on the substrate, enzyme, or regulatory factor is complicated by the fact that all of the proteins interact with Ca^{2+}. Previous work from Dr. Esmon's laboratory demonstrated that in protein C activation, Ca^{2+} interaction with the substrate is critical to activation. To determine if other substrates undergo similar changes, activation of factor X was analyzed with a thrombin mutant having altered primary substrate specificity, E192Q. Since thrombin does not bind Ca^{2+}, any changes in activation would reflect alterations in the substrate. These studies indicated that factor X, like protein C, undergoes conformational changes at or near the scissile bond that alter protease sensitivity, presumably contributing to productive interaction with the physiological activation complexes.

Indirect experiments suggested that Ca^{2+} interaction with the first of the two EGF-like domains of protein C is probably critical to the activation process. To test this hypothesis directly, a deletion mutant was expressed and isolated lacking the entire amino-terminal region of protein C and this EGF domain. The resultant molecule binds Ca^{2+}, and the activation process is still Ca^{2+} dependent, suggesting that although the first EGF domain can bind Ca^{2+}, it is not the critical site involved in protein C activation. Grants from the National Institutes of Health helped support all of the work described above.

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CELL SIGNALING THROUGH PHOSPHOLIPID BREAKDOWN

JOHN H. EXTON, M.D., PH.D., *Investigator*

G Protein–mediated Regulation of Phosphoinositide Phospholipase C

Many hormones and neurotransmitters exert their effects by stimulating the breakdown of plasma membrane phosphatidylinositol 4,5-bisphosphate (PIP_2) in their target cells, through activation of a specific phospholipase C to yield inositol 1,4,5-trisphosphate (IP_3), which releases Ca^{2+} ions from internal stores, and 1,2-diacylglycerol (DAG), which activates protein kinase C.

Key components in the signaling system are the hormone receptors, the PIP_2-specific phospholipase C, and the specific GTP-binding regulatory protein (G protein) that links these. Dr. Exton and his colleagues have identified two G proteins from bovine liver plasma membranes that specifically regulate the β_1-isozyme of phospholipase C and have purified them to homogeneity in both the α-subunit and heterotrimeric ($\alpha\beta\gamma$) forms. The heterotrimeric proteins have α subunits of 42 and 43 kDa, which have been identified by sequencing as α_q and α_{11}, two novel G protein α subunits identified by molecular cloning by Dr. Melvin Simon (California Institute of Technology).

Studies of the PIP_2 phospholipase C isozyme specificity for activation by the G proteins have indicated unequivocally that the β_1-isozyme is activated by both the 42- and 43-kDa α subunits, whereas the γ_1- and δ_1-isozymes are not. This is different from the isozyme specificity for phosphorylation by growth factor receptor tyrosine kinases.

In collaborative studies with Dr. Elliott Ross (University of Texas Southwestern Medical Center at Dallas), Dr. Exton and his colleagues have reconstituted G_q and G_{11} purified from bovine liver with recombinant M1 muscarinic receptor in lipid vesicles and have demonstrated cholinergic agonist activation of the G proteins demonstrated by stimulation of the binding of the labeled GTP analogue [^{35}S]GTPγS. In contrast, the M2 receptor was a poor activator of $G_{q/11}$. The activation of GTPγS binding to $G_{q/11}$ induced by agonist in the presence of the M1 receptor was paralleled by an increased ability of the G proteins to activate the β_1-isozyme of PIP_2 phospholipase C. Co-reconstitution of this receptor with $G_{q/11}$ and the phospholipase demonstrated guanine nucleotide–dependent agonist activation of PIP_2 hydrolysis.

During these studies, the GTPase activity of $G_{q/11}$ was low relative to that of most other G proteins, even when activated by agonist and receptor. This activity could be greatly stimulated by phospholipase C-β_1 in the presence of the M1 receptor and cholinergic agonist. These studies show that the phospholipase C acts as a GTPase-activating protein (GAP) for its G protein regulator $G_{q/11}$. This would provide a mechanism for the rapid termination of agonist activation of the lipase.

In a search for other G proteins that activate other PIP_2 phospholipase C isozymes, a novel cytosolic phospholipase was found in liver and brain that was not related to the other known (β, γ, δ) isozymes. This was stimulated by a mixture of G proteins activated by GTPγS. Purification of the G protein subunit responsible for the stimulation revealed the surprising finding that it was the $\beta\gamma$ complex. Although this complex produced a very large stimulation of the soluble phospholipase, it was ineffective on the δ_1- and γ_1-isozymes and produced a small stimulation of the β_1-isozyme. A 25-nanomolar concentration of brain or liver $\beta\gamma$ was necessary for half-maximal stimulation of the soluble enzyme. The activation was completely reversed by excess α_o. The findings demonstrate a novel mechanism of G protein activation of a phospholipase, namely via the $\beta\gamma$ complex.

Regulation of Phosphatidylcholine Hydrolysis

Many growth factors and other agonists stimulate the breakdown of phosphatidylcholine (PC) in cells, through activation of phospholipase C and D. The products are DAG and phosphatidate (PA). One target of DAG is protein kinase C, but this consists of many isozymes (α–η). The activation and translocation of specific isozymes in response to PC and PIP_2 breakdown induced by α-thrombin is being studied in IIC9 fibroblasts. The function(s) of PA remains obscure, but one possible target is protein kinase Cζ. This enzyme was previously identified only at the cDNA level, and its properties were not well defined. Its purification from several mammalian tissues was undertaken, and it was finally purified to homogeneity from bovine kidney. Its molecular weight at 78,000 and its kinetic properties and insensitivity to Ca^{2+} were established. More importantly, it was shown not to bind phorbol esters or respond to these compounds. However, it responds to certain lipids, including phosphatidylserine, PA, and unsaturated fatty acids. Potential mechanisms of control of this ubiquitous kinase are being studied.

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Despite much indirect evidence that protein kinase C is a major factor in the control of PC hydrolysis by many hormones and growth factors, direct effects of the kinase on PC breakdown have not been demonstrated. Plasma membranes from CCL39 fibroblasts were used to stimulate PC phospholipase D activity by the dose-dependent addition of purified protein kinase C and phorbol ester. Addition of either alone was without effect. Surprisingly, addition of ATP was not required, and the inclusion of an ATPase did not diminish the effect. Furthermore, activation of phospholipase D could be demonstrated with protein kinase C plus phorbol ester in the total absence of histone phosphorylation or autophosphorylation of the kinase. These results establish that protein kinase C can regulate phospholipase D, but phosphorylation is not involved: i.e., the regulation involves a protein-protein interaction. The specific protein kinase C isozymes and the specific protein domains involved are being studied.

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MOLECULAR BIOLOGY OF THE SKIN

ELAINE FUCHS, PH.D., *Investigator*

The global objective of this laboratory is to understand the molecular mechanisms underlying epidermal differentiation and development. Epidermal cells manifest their protective function by building an extensive cytoskeletal architecture of 10-nm keratin filaments (intermediate filaments, IFs), which account for 30% of basal cell protein and 85% of protein in fully differentiated squamae. As keratinocytes commit to differentiate, they switch off expression of K5 and K14, which form dispersed filaments, and switch on expression of K1 and K10, which assemble into filaments that bundle. This progressive increase in filament bundling seems to enable IFs to be among the few survivors of the massive destructive phase that ensues as a cell becomes metabolically inert and reaches the skin surface. A knowledge of the function and structure of keratin IFs and how keratin genes are regulated is prerequisite to understanding epidermal differentiation and the possible involvement of keratin mutations in genetic disease.

Function and Structure of Keratin Filaments, and Relevance to Genetic Skin Disorders

Approximately 10,000 coiled-coil heterodimers of type I and type II keratins form stable heterotetramers that then self-assemble into each IF. To elucidate this basic subunit structure and to determine the mechanisms underlying assembly, this laboratory conducted deletion and site-directed mutagenesis of cDNAs encoding human K5 and K14. For *in vitro* filament assembly studies, mutant and wild-type keratin cDNAs were overexpressed in bacteria, and biochemical methods were used to purify milligram quantities of human keratins. Biochemical and electron microscopic studies revealed that 1) the nonhelical tail domains play a role in filament stabilization, 2) the nonhelical head domain of K5 and the central α-helical rod segments of K5 and K14 are essential for IF structure, 3) rod-deletion mutants interfere predominantly with end-to-end rather than with lateral interactions, and 4) even subtle point mutants in the highly conserved rod ends are more deleterious to IF assembly than are proline mutations within the rod. These studies were the first to use molecular genetics to examine IF assembly in detail.

Dr. Fuchs and her colleagues pioneered methods to examine effects of IF deletions on cytoskeletal networks of cultured cells. They then prepared transgenic mice expressing severe and mild IF-disrupting mutants of the human K14 gene. Surprisingly, these mice exhibited a striking resemblance to a group of human blistering skin diseases known as epidermolysis bullosa simplex (EBS). Upon mild physical trauma, their skin blistered because of cytolysis of basal epidermal cells. Mice expressing severely disrupting K14 mutants resembled the most severe form, Dowling-Meara EBS, with large clumps of keratin in the cytoplasm of their basal cells. Mice expressing milder K14 mutants resembled milder forms of EBS, with disorganization but no gross clumping of keratin protein. These results suggest strongly that 1) EBS arises from defects in the formation of K5/K14 filament networks, and 2) the multiple forms of EBS are genetically related, arising from different defects in the K14/K5 genes. Finally, the correlation between perturbation of keratin IFs and cell cytolysis on mechanical stress indicates that an important function of keratin IFs is to impart mechanical integrity to epidermal cells.

A major focus of last year was to isolate and characterize the K14 and K5 cDNAs/genes from patients with EBS. In two distinct incidences of Dowling-Meara EBS, point mutations were found in the K14 gene, in a codon for a highly conserved arginine residue at the amino terminus of the K14 rod domain. Using genetic engineering, gene transfection, and *in vitro* filament assembly, Dr. Fuchs and her colleagues showed that these mutations are functionally responsible for the basal cell tonofilament clumping that is a hallmark of the disease.

This research on EBS suggests that a second genetic skin disease, epidermolytic hyperkeratosis (EH), might be a disorder of the differentiation-specific keratins K1 and K10. EH is characterized by tonofilament clumping and cytolysis in suprabasal rather than in basal cells. To address this possibility, a truncated version of the human K10 gene was generated. In mice this mutant caused the clinical and biochemical manifestations of EH. These findings prompted an investigation of human EH, and recently Dr. Fuchs and her colleagues discovered a point mutation in the human K10 gene of two unrelated families with EH. The mutation is present only in affected family members, and subsequent analysis demonstrated that it is functionally responsible for the clinical manifestations of EH in these patients.

Regulation of Keratin Gene Expression

As agents for targeting expression of foreign genes in skin and for altering expression patterns of genes involved in growth and differentiation, the promoters of epidermal keratin genes will be invaluable. This laboratory isolated and characterized the functional genes encoding K5 and K14. They showed that K5/K14 transcriptional rates in keratinocytes are high, and they have partially characterized sequences directing proper tissue-specific expression.

Two K14 regulatory elements were identified that act synergistically with a TATA box to drive expression in keratinocytes *in vitro* and in transgenic mice. Analyses of the proximal domain revealed an essential 10-bp palindrome that specifically binds the nuclear factor AP-2. AP-2 is prevalent in epidermal and neural cells, and AP-2 sites occur in promoter regions of many epidermal genes. The distal element of the K14 gene is more complex, containing both positive and negative elements, and studies are under way to analyze these elements. Parallel studies are in progress to delineate sequences responsible for human K5 gene expression, and it is already clear that many of the same regulatory elements are shared by these coexpressed genes. Additionally, studies have been initiated to dissect out elements involved in keratin gene switching during differentiation. Because 1) skin from founder transgenic mice can readily be biopsied and analyzed, 2) skin contains ~20 different cell types, 3) pure epidermis can readily be separated from the rest of skin, and 4) K5 and K14 genes are the major structural proteins of basal keratinocytes, these genes are among only a few that are particularly amenable to an *in vivo* study of tissue-specific and differentiation-specific gene expression.

Regulators of Epidermal Growth and Differentiation *in Vitro* and *in Vivo*

Previously, this laboratory optimized conditions for cultivation of human epidermal cells, squamous cell carcinomas, and human papillomavirus-transfected keratinocytes such that most of their differentiative and altered epidermal phenotypes are maintained. These researchers have utilized this system to examine the effects of TGF-α (transforming growth factor-α), TGF-βs, retinoids, KGF (keratinocyte growth factor), TNF-α (tumor necrosis factor-α), and interleukins on keratinocyte growth and differentiation. Of special interest is the keratinocyte autocrine growth factor TGF-α, whose levels are higher in skin from patients with psoriasis, squa-

mous cell carcinomas, and other hyperproliferative skin diseases. The laboratory has used the K14 promoter to target overexpression of TGF-α to the basal layer in transgenic mouse skin. These studies revealed that 1) TGF-α is involved in calibrating epidermal thickness, a phenomenon that appears to be mediated by epidermal growth factor (EGF) receptor levels; 2) at high levels, TGF-α can bypass the need for a tumor initiator in generating wound- or tissue plasminogen activator (TPA)-induced papillomas; and 3) TGF-α generates some but not all of the features of psoriasis.

In the past year this group has begun to follow up on these observations. Their findings include demonstrations that 1) TPA-induced papillomas in TGF-α mice do not have mutations in Ha-*ras*, as do chemically induced papillomas; and 2) two other factors, interleukin-6 (IL-6) and TNF-α, elevated in psoriatic epidermal cells, can elicit in transgenic mice certain responses typical of psoriasis that are not induced by TGF-α. The demonstration that the K14 promoter can be used to target expression of genes to stratified squamous epithelia has set the foundation for a powerful approach to examine the molecular basis for a number of different human skin diseases and to provide important animal models for the treatment of these diseases.

Dr. Fuchs is also Professor of Molecular Genetics and Cell Biology and of Biochemistry and Molecular Biology at the University of Chicago.

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VIRAL REPLICATION AND PATHOGENESIS

Donald E. Ganem, M.D., *Associate Investigator*

Dr. Ganem's laboratory is interested in the molecular mechanisms by which human and animal viral pathogens replicate in their hosts and cause disease. Work of this group centers around the hepatitis B viruses (hepadnaviruses), small hepatotropic DNA viruses that produce acute and chronic liver injury and are strongly associated with the development of liver cancer.

Hepadnaviruses and Liver Cancer

Persistent infection from birth with human hepatitis B virus (HBV) results in a 100-fold increase in the risk of developing hepatocellular carcinoma (HCC). The molecular mechanism by which HBV engenders HCC is unknown and constitutes one of the great unsolved puzzles of human cancer biology. Tumors typically arise ~30 years after the onset of infection. In most tumor samples, active viral replication (often seen in the surrounding nontumorous liver) is not observed; instead, integrated copies of the HBV genomes are found. Tumors are clonal with respect to these insertions, indicating that integration preceded or coincided with the final transforming event leading to outgrowth of the mass.

Opinion is divided as to the importance of these integrants in the pathogenesis of human HCC. Certainly one possible role for such integrants is in the disruption or activation of flanking host genes.

Work in Dr. Ganem's laboratory and in the group of Dr. Marie-Annick Buendia in Paris has established a central role for viral integration in the pathogenesis of HCC in infection by woodchuck hepatitis virus (WHV). WHV is a close animal relative of HBV that shares with it DNA sequence homology, antigenic cross-reactivity, and many key biological features. WHV induces HCC in 100% of animals infected from birth, with tumors beginning in young adulthood. About 40% of these tumors can be shown to harbor viral insertions flanking the N-*myc* cellular proto-oncogene. This gene is normally silent in adult liver, but viral insertion leads to the accumulation of abundant N-*myc* mRNA. Insertions generally occur within 2–3 kb of the gene but can be either 3' or 5' to the locus.

N-*myc* loci activated this way are now being cloned, with the plan being to establish them in transgenic mice, thereby providing a system to prove that these rearrangements are on the pathway to HCC formation. Attempts are also being made to determine if other cellular loci are regularly disrupted by viral integration: the goal is to use WHV sequences as a "transposon tag" to clone other host loci involved in the control of hepatocyte proliferation.

Another mammalian virus, the ground squirrel hepatitis virus (GSHV), also induces HCC in its natural host, although with a lower incidence and with

greatly delayed kinetics. This virus also infects woodchucks, allowing determination of whether host or viral factors control these biological differences. Studies of GSHV-infected woodchucks demonstrated that GSHV was potently oncogenic, but again tumors appeared only late in life. Moreover, molecular analysis of tumor DNA revealed that N-*myc* loci are not rearranged in these tumors. Attempts to define potential targets of GSHV insertion in these tumors are now under way.

Viral Replication

Work also continues on the molecular basis of hepadnaviral replication. This process is of great intrinsic interest because the replication of these DNA viruses proceeds through reverse transcription of an RNA intermediate. This reaction is formally analogous to that involved in retroviral replication but differs mechanistically in many major ways. A fuller understanding of this process should inform studies of other reverse transcription reactions and may also define novel potential targets for antiviral therapy.

Replicative intermediates in hepadnaviral replication have been identified through the study of viral mutants blocked at various stages of minus- and plus-strand DNA synthesis. These studies have led Dr. Ganem and his colleagues to the first mechanistic understanding of how duplex linear viral DNA is generated and how the RNA primer for plus-strand synthesis is cleaved from genomic RNA. The latter is accomplished not by recognition of specific sequences, as had previously been thought, but by measurement of the distance from the 5' end of the transcript.

The genomic RNA of hepadnaviruses is terminally redundant, and each copy of the redundancy harbors potential polyadenylation signals. Thus synthesis of full-length RNA requires that the 5' poly(A) signals be bypassed, while the 3' signals are efficiently utilized. Experiments addressing this interesting form of regulation revealed that positioning of the poly(A) signals close to a cap site greatly suppresses their recognition; the mechanism of this suppression is unknown. Other, more complex, elements also contribute (to a lesser degree) to this regulation.

Once replication is complete, progeny viral nucleocapsids bud into the endoplasmic reticulum, thereby acquiring their complement of the three envelope glycoproteins L, M, and S. Mutant viruses bearing lesions in each coding region were constructed and examined, to explore the functions of these proteins in viral assembly. Only the L and S proteins were required for assembly and secretion; the function of the M protein remains a mystery.

Another role of the envelope proteins is to bind the cellular receptor for the virus, thereby initiating viral entry. In recent work, cellular polypeptides that bind viral envelope proteins have been sought by biochemical assays. In this way a host glycoprotein of 180 kDa has been identified that binds the L protein of the avian hepatitis B virus with high affinity. Binding can be blocked by neutralizing monoclonal antibodies. Clones for this protein have been obtained and their characterization is under way.

Work on hepadnaviral replication is supported by the National Institutes of Health.

Dr. Ganem is also Professor of Microbiology and Immunology and of Medicine (Infectious Diseases) at the University of California, San Francisco.

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SECOND MESSENGERS AND CELL REGULATION

David L. Garbers, Ph.D., *Investigator*

Spermatozoan behavior is altered in various ways when the cells are in the vicinity of the egg. In experiments that targeted the molecular basis of signaling between the egg and the spermatozoon in the sea urchin, Dr. Garbers' laboratory found that one of the sperm receptors was the enzyme guanylyl cyclase. Subsequently members of the same receptor family were discovered in mammalian somatic cells. Three particulate forms are currently known to exist in mammals and are designated GC-A, GC-B, and GC-C, in the order of the cloning of their mRNA. At least two other particulate forms are also known to exist. There are also at least four subunits of guanylyl cyclase that combine to form active heterodimers, designated $\alpha 1$, $\alpha 2$, $\beta 1$, and $\beta 2$. The normal pairing of the subunits is not known, except for $\alpha 1/\beta 1$, where the heterodimeric protein has been purified from lung. During the past year, research in Dr. Garbers' laboratory has focused on the function of these guanylyl cyclase–linked receptors.

Phenotype of the Expressed GC-C Receptor

After the stable expression of GC-C in human embryonic kidney 293 cells, cGMP concentrations were elevated ~ 40-fold in response to heat-stable enterotoxins (STa). STa stimulated the cyclase of isolated membranes from these cells about ninefold. ATP appears to potentiate the STa stimulation, but unlike the situation with GC-A, the nucleotide appears to protect GC-C against inactivation. Antibody prepared to the carboxyl-terminal peptide of GC-C immunoprecipitated two proteins of molecular weight 140,000 and 160,000 from the 293 cells. Tunicamycin treatment of the cells caused both proteins to collapse to a molecular weight of 120,000, the molecular size predicted from the cDNA sequence. Therefore GC-C is an amino-linked glycoprotein. In preparations of rat intestinal membranes, three major proteins of molecular weight 65,000, 85,000, and 140,000 were specifically recognized by the GC-C antibody on reducing SDS-PAGE (sodium dodecyl sulfate–polyacrylamide gel electrophoresis), but only a single protein of molecular weight 230,000 was recognized under nonreducing conditions. Therefore GC-C is apparently proteolyzed in the intestine but does not dissociate because of the presence of inter- and intramolecular disulfide bonds. It can be concluded that many or all of the lower-molecular-weight STa-binding proteins previously reported in intestinal extracts represent proteolytic products of GC-C.

Cloning and Expression of the cDNA for Guanylin

A small peptide named guanylin was recently isolated from the intestine of the rat and shown to elevate cGMP and to compete with STa for receptor binding. In studies designed to determine whether guanylin represents the endogenous ligand for GC-C, the cDNA encoding this peptide was cloned. The mRNA encodes an open reading frame of 115 amino acids; the 15-residue guanylin was found at the carboxyl terminus. The peptide initially isolated likely represents an isolation artifact, however, because an acid-labile Asp/Pro bond exists at the amino terminus. Transfection of COS-7 cells with the guanylin cDNA yielded a secreted protein with a molecular weight of $\sim 10,000$, but the expressed proguanylin failed to stimulate GC-C unless it was cleaved to yield lower-molecular-weight peptides. Guanylin mRNA was detected in the intestine, kidney, adrenal gland, and uterus/oviduct of the rat by Northern hybridization. GC-C, the apparent receptor for guanylin, was detected in the adrenal gland, airway epithelial cells, brain, and olfactory and tracheal mucosa by Northern analysis, the polymerase chain reaction, or cDNA cloning. Therefore guanylin and its putative receptor exist in various mammalian tissues.

Mechanisms of Guanylyl Cyclase Desensitization

When GC-A, the receptor for atrial natriuretic peptide (ANP), was stably expressed in human embryonic kidney 293 cells, cellular cGMP concentrations were transiently elevated by ANP. GC-A was subsequently shown to exist as a phosphoprotein under basal conditions, and the addition of ANP caused a decrease in ^{32}P content. Coincident with the loss of ^{32}P, GC-A showed a marked reduction in ANP-stimulable activity. The addition of protein phosphatase 2A also caused both the disappearance of ^{32}P from GC-A and a loss of responsiveness to ANP, suggesting that GC-A is desensitized by dephosphorylation.

Mechanisms of Signal Transduction by Guanylyl Cyclases

Transduction of the binding signal in growth factor receptors appears to be associated intimately with a ligand-induced aggregation of receptors. The guanylyl cyclase receptors are similar to growth factor receptors in the sense they are about the same

length, contain a single transmembrane domain, and contain a protein kinase–like domain just within the transmembrane region. A deletion mutant containing the cyclase catalytic domain and only a small part of the protein kinase domain contained guanylyl cyclase activity. However, when analyzed on gel permeation columns, activity was only observed at the position of the homodimer. The chaotropic salt sodium trichloroacetate dissociated the dimer into a monomer and also destroyed activity. The full-length receptor migrated principally as a monomer on SDS-PAGE, but the prior incubation of membranes with ANP or with ANP and ATP resulted in the conversion of a small fraction of the monomer to a disulfide-linked dimer. Subsequent crosslinking experiments showed that GC-A exists as a dimer or higher-ordered oligomer in the absence of ANP. Therefore ANP does not induce receptor oligomerization as seen with the growth factor receptors but likely causes conformational changes in an already existent dimer or tetramer.

Dr. Garbers is also Professor of Pharmacology at the University of Texas Southwestern Medical Center at Dallas.

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MOLECULAR ANALYSIS OF PROTEINS INVOLVED IN HUMAN DISEASE

MARY-JANE H. GETHING, PH.D., *Investigator*

Investigations in this laboratory of experimental models of human disease grow out of several years of basic research on the biochemical and structural properties of cellular and viral proteins. Studies supported by HHMI involve three systems: 1) human tissue-type plasminogen activator (t-PA), a serine protease involved in fibrinolysis, tissue remodeling, and metastasis; 2) the hemagglutinin (HA) of influenza virus, which is being used to develop models of autoimmune disease in transgenic mice; and 3) the tumor-suppressor protein p53 and its interaction with cytosolic stress-70 proteins. In addition, basic studies on the cellular role and regulation of protein chaperones are supported by the National Institutes of Health.

Role of Tissue-Type Plasminogen Activator in Thrombolysis and Metastasis

Many normal and abnormal biological processes that require extracellular proteolysis, including thrombolysis, tissue remodeling, and metastasis, are mediated by plasminogen activators that cleave plasminogen to the active protease plasmin. The t-PA is the principal thrombolytic agent in the circulation; elevated expression of t-PA is thought to be linked to increases in metastatic potential of some types of tumor cells, including malignant melanomas.

The t-PA protein is composed of a number of independent structural domains that are encoded by individual exons in the t-PA gene. The finger and epidermal growth factor (EGF)-like domains are involved in the initial, high-affinity binding of t-PA to fibrin, whereas stimulation of t-PA activity requires secondary, lower affinity interactions of fibrin with either of the two kringle domains of the molecule. The binding of t-PA to specific clearance receptors on hepatic cells also involves sequences within the finger and/or EGF-like domains. Finally, the specific inhibitor plasminogen activator inhibitor-1 (PAI-1) interacts with the active site in the carboxyl-terminal catalytic domain.

Although the three-dimensional structure of t-PA has not been elucidated, Dr. Gething and her colleagues have been able to model all the domains, using the known structures of homologous domains in other proteins. Site-directed mutants designed using these proposed structures have provided information about the role of individual amino acid sequences of t-PA. Variant enzymes have been generated that are efficient, fibrin-stimulated plasminogen activators but are resistant to inhibition by a variety of serpins (including PAI-1) or do not bind to the t-PA receptor(s) involved in clearance of the enzyme in the liver. Because these mutant enzymes should have an extended effective life in the circulation, they may have significant potential for use in thrombolytic therapy of patients with myocardial infarction.

The variant enzymes are also being utilized to test the role of t-PA in metastasis of malignant melanoma cells. Transgenic mice expressing simian virus 40 (SV40) T antigen from the tyrosinase promoter develop nonmetastatic melanoma with high frequency. Orthotopic translation of these tumors to nontransgenic animals results in progressive growth but not metastasis. Similar transplantation into nude mice, however, results in a frequent occurrence of metastasis to the liver, as is seen in human ocular melanoma. Current experiments involve investigations of the mechanisms by which these tumors acquire metastatic potential by crossing these animals with other transgenic mice that express t-PA (wild-type or inhibitor-resistant forms) or PAI-1 from the same tyrosinase promoter.

Transgenic Models of Autoimmune Disease

Dr. Gething and her colleagues are using transgenic mice that express the HA glycoprotein of influenza virus to study the mechanisms underlying the development of autoimmunity. RIPHA mice, which express HA from the rat insulin II promoter/enhancer only in the β cells of the pancreas, can develop an immune response to HA and other antigens of pancreatic β cells. However, the immune response is not as penetrant or as active as that observed in non-obese diabetic (NOD) mice, and current studies focus on a comparison of these two models. To compare the specific immune response to the well-characterized HA antigen in the two lines, the RIPHA gene has been introduced onto the NOD genetic background by crossing of RIPHA and NOD mice followed by extensive backcrossing to NOD. This study is being complemented by an analysis of the role of the inflammatory cytokines interleukin-1 (IL-1), tumor necrosis factor (TNF), and IL-6 during autoimmune diabetes. Re-

cent studies by others indicate that IL-1 and TNF can inhibit the onset of diabetes in NOD mice, probably via immunoregulation. The effects of immunostimulating RIPHA and RIPHA/NOD mice with influenza virus will be examined.

Role of Stress-70 Proteins in Folding and Assembly of the Tumor-Suppressor Protein p53

Until recently it was widely assumed that the folding and oligomerization of newly synthesized polypeptides and their subsequent molecular rearrangements are spontaneous processes that do not require the intervention of other cellular proteins. However, it is now apparent that members of the stress-70 (hsp70; heat-shock protein 70) protein family are intimately involved in facilitating protein folding and assembly within prokaryotic and eukaryotic cells.

Dr. Gething's previous studies of the interaction of the endoplasmic reticulum (ER) stress-70 protein BiP with newly synthesized membrane and secretory proteins has led to an understanding of the role of stress-70 proteins in stabilizing unfolded or partly folded polypeptides in a form competent for further folding and oligomeric assembly. Current studies focus on the role of the cytosolic stress-70 protein hsc70 in modulating the structure or activity of mutant forms of the tumor-suppressor protein p53. Others have shown that p53 plays a role in regulation of normal cell growth and that it binds to a number of viral transforming proteins, including SV40 T antigen. Mutations in p53 convert the protein to an oncogenic form capable of cooperating with activated *ras* to transform cells. These mutant forms of p53 appear to be altered conformationally, as indicated by loss of T antigen recognition, altered reactivity with monoclonal antibodies, and cotranslational binding to hsc70.

Dr. Gething's group is using the fd bacteriophage expression system to analyze the interaction between p53 and hsc70. Oligonucleotide sequences encoding peptides from wild-type and mutant forms of p53 have been synthesized as amino-terminal fusions to the phage adsorption protein pIII and expressed on the surface of phage particles. By screening p53-peptide–expressing phage for binding to hsc70, with subsequent enrichment and amplification in bacteria of those expressing hsc70-binding epitopes, the region(s) in p53 recognized by hsc70 will be identified.

Protein Folding in the ER: Role of BiP

In the cell, as *in vitro*, the final conformation of a protein is determined by its amino acid sequence.

But whereas some isolated proteins can be denatured and refolded *in vitro* in the absence of other macromolecular cellular components, folding and assembly of polypeptides *in vivo* involves other proteins, many of which belong to families that have been highly conserved during evolution. These proteins, known as chaperones, are involved at all stages of cellular metabolism: during protein synthesis, membrane translocation, and folding; in rearrangements of macromolecules during functional cycles of assembly and disassembly; in protection from environmental stress; and in targeting proteins for degradation.

ER chaperone BiP provides an ideal system to study in detail the interactions between one chaperone and its substrates. BiP binds transiently to newly synthesized wild-type polypeptides and more permanently to malfolded or unassembled proteins. Under normal growth conditions BiP is synthesized constitutively and abundantly, comprising ~5% of the luminal content of the ER. However, its synthesis can be further induced by the accumulation of mutant proteins in the ER or by a variety of stress conditions whose common denominator is likely to be the generation in the ER of misfolded polypeptides. The overall goals of this investigation are 1) to determine how BiP discriminates between folded and unfolded structures and what role it plays during protein folding and 2) to elucidate the pathway of induction of the BiP gene in response to the accumulation of unfolded proteins. Three experimental systems are being utilized: mammalian cells for analysis of *in vivo* interactions between BiP and its protein substrates, *Escherichia coli* for expression of BiP proteins and of peptide libraries for *in vitro* binding studies, and the yeast *Saccharomyces cerevisiae* for genetic analysis of the pathway of induction of BiP synthesis.

Dr. Gething is also Professor of Biochemistry at the University of Texas Southwestern Medical Center at Dallas.

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MEMBRANE LIPIDS AND THE REGULATION OF CELL FUNCTION

John A. Glomset, M.D., *Investigator*

Current research in Dr. Glomset's laboratory is focused on the lipids of animal cells. In one set of studies, structural determinants that affect the interactions of phosphorus-containing lipids in cell membranes are being examined at a molecular level. A recently completed, computer-based molecular modeling study of the effects of phospholipid polyunsaturated fatty acids on these interactions led to three major findings: 1) the presence of multiple cis double bonds in a polyunsaturated fatty acid chain need not prevent that chain from adopting a straight conformation; 2) the presence of an esterified, polyunsaturated fatty acyl chain in a lipid need not interfere with that lipid's ability to pack with similar lipids in tightly packed monolayers; and 3) the precise packing geometry of polyunsaturated lipid molecules in a monolayer can be a function of the location of the sequence of cis double bonds within the polyunsaturated fatty acyl chain. Together these findings provide important clues concerning factors that may influence the domain structure of animal cell membranes. Therefore, experimental tests of the models with well-characterized phospholipids are under way, in

collaboration with Dr. Howard Brockman (Hormel Institute).

In a second set of studies, a newly discovered pathway of animal cell phospholipid biosynthesis is being characterized. Recent experiments in Dr. Glomset's laboratory showed that animal cells in culture readily convert sn-2-arachidonoyl monoacylglycerol into sn-1-stearoyl-2-arachidonoyl species of several membrane phospholipids, including phosphatidylinositol, phosphatidylethanolamine, and phosphatidylserine. In addition, experiments with cell-free systems provided evidence that a monoacylglycerol kinase activity initiates the monoacylglycerol incorporation pathway by converting monoacylglycerol into sn-2-acyl lysophosphatidic acid and that a stearoyl-specific, coenzyme A–dependent transacylase activity catalyzes the next step in the pathway by converting sn-2-acyl lysophosphatidic acid into sn-1-stearoyl-2-acyl phosphatidic acid. Subsequent experiments with a similar transacylase activity from bovine testis membranes provided more-detailed information about the enzyme's substrate specificity and mechanism of action. Attempts to purify the enzyme and determine its intracellular location are under way, as are attempts to obtain more-detailed information about other enzymes in the pathway.

A third set of studies (in collaboration with Dr. Andreas Habenicht, University of Heidelberg) is designed to provide information about metabolic pathways that deliver arachidonic acid for eicosanoid formation in animal cells. An initial study of human skin fibroblasts in culture demonstrated that the classical, low-density lipoprotein (LDL) receptor-dependent pathway of LDL uptake delivers arachidonic acid for eicosanoid production during the cell cycle; a recent study showed that the LDL receptor-dependent pathway also provides arachidonic acid for eicosanoid production in freshly isolated, human blood-derived monocytes. Importantly, the monocytes convert the arachidonic acid into products of the prostaglandin H synthase pathway, even in the absence of agonists that increase the concentration of intracellular calcium ions. In contrast, they convert the arachidonic acid into products of the 5-lipoxygenase pathway only in the presence of such agonists. Experiments are currently under way to determine whether an LDL receptor-dependent pathway of arachidonic acid delivery also operates in other cells, such as those of the adrenal cortex.

A fourth set of studies concerns membrane proteins that are post-translationally modified by one or more thioether-linked, 20-carbon isoprenoid groups (geranylgeranyl groups). Previous studies by

Dr. Glomset's group in collaboration with others showed that several of these proteins, including the proteins rab 3A and G25K (CDC42Hs), are members of the ras superfamily of low-molecular-weight GTP-binding proteins. Studies of the mechanism of binding of these proteins to specific cell membranes are currently in progress.

Dr. Glomset is also Professor of Medicine and of Biochemistry at the University of Washington School of Medicine, Seattle, and Core Staff Member of the Regional Primate Research Center at the University of Washington.

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CELL DIFFERENTIATION AND DENSITY SENSING

RICHARD H. GOMER, PH.D., *Assistant Investigator*

Little is known about the molecular mechanisms that regulate the differentiation of cells into various cell types or the mechanisms that allow cells to sense how many of a given cell type are in an organism. Dr. Gomer's laboratory is studying cell-type differentiation and density-sensing mechanisms used by the simple eukaryote *Dictyostelium discoideum*.

Dictyostelium normally grows as isolated amoebae that multiply by fission. In the wild, the amoebae feed on bacteria on decaying leaves and the soil surface. When the cells starve, they stop dividing and aggregate using relayed pulses of cAMP as the chemoattractant. The cells form aggregation streams that flow and coalesce into an aggregation center. The aggregate then elongates into a slug that uses phototaxis and thermotaxis to crawl to an open area, whereupon it differentiates into a fruiting body consisting of a mass of spore cells held up by an ~2-mm column of stalk cells. Spores can then be dispersed by the wind or insects to start new colonies of *Dictyostelium*.

Development of Techniques for Gene Isolation

The initial decision for a *Dictyostelium* cell to differentiate into a precursor of either a spore or stalk cell is based on the cell-cycle phase of each cell at the time of starvation. For cells that are starved in S or early G_2 phase (*Dictyostelium* does not have a G_1 phase), one sister differentiates into a prestalk cell, while the other sister becomes a null cell (a cell that does not express either a characteristic prestalk or a prespore antigen). Cells starved in late G_2 or M phase similarly differentiate into either a prespore or a null cell.

Until recently there have been no general methods available to identify the gene causing a given mutation in *Dictyostelium*. Using available techniques for transformation, homologous recombination, gene disruption, and antisense repression, Dr. Gomer's laboratory has begun to develop general methods to identify genes in *Dictyostelium*, and to use the methods specifically to isolate genes involved in the cell-cycle-dependent cell-type differentiation mechanism. Using aggregate and fruiting body morphology for the initial screen, three different methods were tried. First, overexpression transformants were generated by using extrachromosomal vectors containing ~10-kb regions of random fragments of genomic DNA; this method has proved to be unsatisfactory.

As a second method, random homologous integration was tried in a manner similar to P-element mutagenesis in *Drosophila* to generate transformants with altered cell-type ratios. Several transformants with abnormal morphologies were identified. These include no aggregation, aggregation but no fruiting body formation, and aggregates with multiple long, string-like projections. Some of these appear to have abnormal prestalk:prespore cell-type ratios, as assayed by immunofluorescence. Dr. Gomer's laboratory has isolated the DNA regions flanking the insertion site from one of these transformants and is testing whether the insertion caused the phenotype.

As a third approach, libraries of cDNA fragments were made for shotgun antisense transformation. Unlike in higher eukaryotes, antisense works well in *Dictyostelium*. From a morphological screen of 2,800 antisense transformants, 25 having unusual developmental morphologies were identified. These include failure to aggregate, failure to proceed beyond aggregation, very small aggregates that do not form fruiting bodies, and tiny fruiting bodies. The antisense DNA was isolated from the 25 transformants, cloned, and used to retransform *Dictyostelium*. Currently, 4 of the 25 specific cDNAs that cause an abnormal developmental morphology have been identified. The success of this technique indicates that it will be a powerful general method to isolate genes rapidly in *Dictyostelium*. For one of the four cloned cDNA antisense transformants, cells aggregate but then do not proceed to form a fruiting body. This transformant has an abnormal cell-type ratio. This cDNA fragment is currently being sequenced, and a larger fragment of the cDNA has been isolated by screening a cDNA library. Dr. Gomer's laboratory is currently identifying additional genes that, when their expression is disrupted, cause an abnormal ratio of cell types.

Density Sensing

During *Dictyostelium* development, the expression of some genes depends on cell density. This effect is mediated by soluble factors referred to as conditioned medium factors (CMFs), which the developing cells secrete at very low rates and simultaneously sense. There are at least two classes of CMFs: one is an 80-kDa glycoprotein; the other is a set of its breakdown fragments, with molecular weights between 6.5 and 0.65 kDa and higher specific activity than the 80-kDa CMF molecule. The two classes of molecules do not need to be combined for activity. Dr. Gomer's laboratory has purified CMF, se-

quenced tryptic peptides, and used degenerate oligonucleotides to isolate a CMF gene. Bacterially synthesized CMF has CMF activity, and like the breakdown peptides, specific bacterially synthesized CMF fragments have even higher activity. This indicates that the cloned cDNA encodes CMF. Using antibodies directed against bacterially synthesized CMF, Dr. Gomer's laboratory has found that CMF is present in approximately the same amount in all *Dictyostelium* cells and is secreted by both prestalk and prespore cells. Diffusion calculations based on the canonical secretion showed that an isolated starved cell does not secrete enough CMF to trigger itself, whereas a high density of starved cells will stimulate themselves.

At the same time that cells are sensing CMF, they begin to use pulses of cAMP as a chemoattractant for aggregation. CMF is required for this cAMP pulse-mediated aggregation. In collaboration with Dr. Peter Van Haastert at the University of Groningen (the Netherlands), Dr. Gomer's laboratory has found that CMF does not affect the binding of cAMP to cell-surface cAMP receptors but does interfere with the ability of the receptors to activate via G proteins two enzymes: adenylate cyclase and guanylate cyclase. These results are helping to explain how CMF allows *Dictyostelium* cells to sense their relative density. The CMF project was supported by a grant from the National Institutes of Health.

Dr. Gomer is also Assistant Professor of Biochemistry and Cell Biology at Rice University and Adjunct Assistant Professor of Cell Biology at Baylor College of Medicine.

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DEVELOPMENTAL CONTROL OF GENE EXPRESSION

Rudolf Grosschedl, Ph.D., *Assistant Investigator*

The process of terminal differentiation turns a multipotential cell into a cell that carries out a particular function or synthesizes a specific product. The lymphoid B cell lineage ultimately generates a cell that secretes antibody. During B cell differentiation, several genes are expressed in a defined cell-type–specific and temporally ordered pattern.

Transcription of the μ immunoglobulin gene encoding the heavy chain (IgH) of the antibody can be detected in virtually all lymphocytes. By contrast, the κ gene encoding the light chain of the antibody is transcribed only in late-stage B cells, and the *mb-1* gene encoding an antibody-associated protein is expressed only in early-stage B cells. The goal of Dr. Grosschedl's research is to gain some insight into the molecular mechanisms that mediate the developmental control of lymphoid-specific gene expression.

Tissue-Specific and Temporal Regulation of Ig Gene Expression in a Transgenic Model

The Ig genes represent one of the best-studied paradigms for cell-type–specific gene expression. Efforts by many laboratories to dissect the regulation of Ig genes in cell culture and *in vitro* allowed for the identification and characterization of several cis- and trans-acting regulatory components. These studies, which were attempts to reduce the complexity of regulation, provided a detailed insight into the properties of individual components. However, they did not indicate how these components are integrated to generate the developmental pattern of μ gene expression observed *in vivo*. Therefore Dr. Grosschedl and his colleagues adopted gene transfer into the mouse germline to examine the contribution of various cis-acting sequences to the regulation of an intact rearranged gene *in vivo*. Spe-

cifically, regulatory sequences that were implicated in the lymphoid-specific expression of the μ gene were mutated in the context of the intact rearranged μ gene.

Previous studies by Dr. Philip Sharp (Massachusetts Institute of Technology) and Dr. Thomas Kadesch (HHMI, University of Pennsylvania) implicated the E5/E2 factor–binding sites in the IgH enhancer in conferring negative regulation in nonlymphoid tissues. Therefore transgenic mice carrying a rearranged μ transgene with point mutations in the E5/E2 factor–binding sites of the enhancer were generated and analyzed. The transgenic mice expressed the E5/E2-mutated μ gene at a high level not only in lymphoid and muscle tissues but also in several nonlymphoid tissues, demonstrating the importance of negative regulation for lymphoid-specific μ gene expression. The mutations of the E5/E2-binding sites, however, did not affect the ''off'' state of μ gene expression in other nonlymphoid tissues. Possibly another tier of negative regulation is involved in actively decreasing the basal level of expression even further.

Regulation of the Accessibility of Nuclear Factor-binding Sites in Native Chromatin

During the past two years, Dr. Grosschedl's laboratory has established an assay to determine the accessibility of an individual factor-binding site in native chromatin and to examine its dependence on regulatory DNA sequences. The assay consists of introducing into the mouse germline a binding site for the prokaryotic RNA polymerase T7, alone or linked to a DNA fragment suspected of conferring accessibility in chromatin. This experimental protocol allows the exogenous DNA to go through the normal developmental processes and to be inserted in the same chromosomal location in various cell types. Subsequently, B-lineage cells (Abelson murine leukemia virus–transformed pre–B cells) and fibroblastic cells are derived from the transgenic mice. The accessibility of the T7 promoter residing in native chromatin is examined by the addition of purified T7 RNA polymerase to isolated nuclei or permeabilized cells and by probing for the appearance of specific T7 transcripts. The use of the T7 promoter as a factor-binding site has the advantage that its recognition by T7 RNA polymerase does not require an interaction with other proteins and allows for a simple readout. Analysis of pre–B cells derived from eight individual transgenic mouse lines carrying the T7 promoter linked to a VP1 reporter gene indicated that the T7 promoter is inaccessible in six lines. In two other lines, the promoter was weakly accessi-

ble. These data suggest that the T7 promoter by itself is primarily inaccessible at various chromosomal locations.

Previous experiments by Dr. Grosschedl's laboratory demonstrated that the μ enhancer is necessary for the expression of a rearranged μ transgene in lymphoid tissues. Moreover, experiments by others indicated that the μ enhancer region can confer transcriptional activity on heterologous transgenes. Therefore the μ enhancer, together with flanking nuclear matrix attachment regions (MARs), was examined for its potential to alter chromatin structure. Analysis of pre–B cells from seven transgenic mouse lines carrying the T7-VP1 gene linked to the μ enhancer/MAR region indicated that the T7 promoter is accessible in all the lines. The number of specific T7 transcripts detected was proportional to the copy number of the transgene, suggesting that the accessibility of the T7 promoter linked to the μ enhancer/MAR region is independent of its chromosomal position. The number of T7 transcripts obtained from the seven μ/MAR-T7 lines was $\sim$10-fold higher than those detected with the two weakly accessible T7 lines and at least 50-fold higher than those obtained from the six inaccessible T7 lines. Together these data indicate that the μ enhancer/MAR region can confer accessibility on a heterologous factor-binding site in native chromatin.

Identification and Characterization of EBF

To unravel some of the mechanisms that allow multiple genes to be expressed at distinct stages of the lymphocyte lineage, Dr. Grosschedl's laboratory included the promoter of the *mb-1* gene in its analysis of cell-type–specific gene regulation. The *mb-1* gene encodes a surface IgM-associated protein and is expressed only in pre–B cells and surface Ig-positive B cells but not in antibody-secreting plasma cells. Isolation and characterization of the *mb-1* promoter in cell culture transfection assays indicated that it directs heterogeneously initiated transcription specifically in early-stage B cells. A functionally important nucleotide sequence in the proximal *mb-1* promoter region was shown to interact with the lymphoid/myeloid lineage-specific protein Pu.1. The Pu.1-binding site in the *mb-1* promoter, however, is also recognized by a ubiquitous factor, raising the question as to how the correct combination of these multiple factors is assembled on the *mb-1* promoter. In addition, Dr. Grosschedl's laboratory has identified a novel pre–B and B cell–specific DNA-binding activity, termed early B cell factor (EBF), that recognizes an upstream *mb-1* promoter sequence. The binding site

for EBF, together with some flanking sequences, was found to confer on a heterologous promoter a pre–B and B cell–specific pattern of activity. Thus the distribution and activity of EBF in various lymphoid and nonlymphoid cell lines parallels precisely the developmental expression pattern of the endogenous *mb-1* gene. A similar DNA-binding activity was also identified by Dr. Harinder Singh's laboratory (HHMI, University of Chicago).

Recently Dr. Grosschedl's laboratory purified EBF to near homogeneity and showed that this DNA-binding activity consists of polypeptides of 65 kDa. Partial amino acid sequences were derived from purified EBF and used to design oligonucleotide primers for the amplification of specific cDNAs from a pre–B cell cDNA library. Overlapping cDNA clones that encoded a protein of 64 kDa were isolated. Three sets of experimental data suggested that the isolated cDNAs encode EBF. First, the gene represented by the cDNA clones is expressed in pre–B and B cells but not in late-stage B cells, T cells, or the nonlymphoid cells examined. Second, the protein encoded by the cDNA has the same DNA-binding specificity as purified EBF. Third, forced expression of the cDNA clone in a nonlymphoid cell line transactivated a reporter gene construct in an EBF-binding site–dependent manner. Analysis of the amino acid sequence of EBF indicated that this lineage-specific protein is encoded by a novel gene with no obvious relative in the current protein database.

Isolation and Characterization of cDNAs Encoding LEF-1

To identify novel lymphocyte-specific regulators of gene expression, Dr. Grosschedl's laboratory isolated, by differential screening of a pre–B cell minus erythroid cell cDNA library, cDNA clones that are expressed specifically in lymphocytes. One of these cDNA clones encodes a pre–B and T cell–specific DNA-binding protein with homology to the chromosomal nonhistone high-mobility group protein HMG-1 and to regulators of cell specialization. The lymphoid-specific DNA-binding protein LEF-1 (lymphoid enhancer-binding factor 1) was found to bind a functionally important nucleotide sequence in the enhancer associated with the T cell antigen receptor (TCR) α gene. The role of LEF-1 in regulating TCR α enhancer function was further shown by transactivation in B cells that normally do not express LEF-1. In collaboration with Dr. Uta Francke (HHMI, Stanford University), the LEF-1 gene was mapped to human chromosome 4 (q23-q25) and mouse chromosome 3 near the *Egf* locus.

The DNA-binding domain of LEF-1 was delineated to a 93–amino acid region, the HMG domain, which has homology to other members of the HMG family of proteins. Several amino acids that are conserved among various members of this family were shown to be important for DNA binding. Analysis of the DNA-binding properties of the HMG domain of LEF-1 indicated that this protein-DNA interaction is characterized by a high binding affinity but modest sequence specificity. Moreover, methylation interference data and replacement of A-T base pairs in the LEF-1–binding site with I-C base pairs suggested that DNA recognition by the HMG domain of LEF-1 occurs primarily in the minor groove of the DNA double helix. Finally, DNA binding by LEF-1 was found to induce a sharp bend of ∼130° in the DNA helix. These hallmarks for DNA binding by the HMG domain of LEF-1 were also extended to the HMG domain of the mammalian testis-determining factor *SRY*.

Dr. Grosschedl's laboratory examined possible regulatory roles of HMG domain–induced DNA bending and found that LEF-1 can function in a manner similar to the bacterial integration host factor (IHF). In a site-specific recombination assay *in vitro*, LEF-1 could functionally replace IHF in its role to align spatially widely separated binding sites for integrase protein. Thus LEF-1 has the potential of facilitating the communication between proteins bound at distant sites and may provide an "architectural" role in the assembly of higher-order nucleoprotein structures.

Dr. Grosschedl is also Associate Professor of Microbiology and Immunology and of Biochemistry and Biophysics at the University of California, San Francisco.

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POLYPEPTIDE HORMONE GENE REGULATION

Joel F. Habener, M.D., *Investigator*

Major emphasis in Dr. Habener's laboratory is presently in two areas: 1) the characterization of DNA-binding proteins responsible for regulated and tissue-specific expression of polypeptide hormone genes and 2) determination of the bioactivities of peptides identified through nucleotide sequencing of precursors encoding peptide hormones.

Nuclear proteins bind to specific DNA sequences in or around the polypeptide hormone genes, to up- or down-regulate the transcription of genes. A major goal is to isolate and characterize the structures and functions of the DNA-binding proteins, with particular regard to the regulation of transcriptional transactivation functions by cellular signal transduction pathways involving protein phosphorylation. Dr. Habener is using pancreatic islet cell lines with distinct hormone-expressing phenotypes to investigate cell-specific expression of the glucagon, somatostatin, insulin, and angiotensinogen genes. Similar studies of expression of the gonadotropin subunit and angiotensinogen genes are being analyzed in placental and liver cell lines, respectively.

Dr. Habener has focused on the cis-control elements and the transacting DNA-binding proteins responsible for the cell-type–specific expression of the glucagon, somatostatin, and insulin genes, because the expression of these polypeptide hormone genes is developmentally regulated during the differentiation of a stem neuroendodermal islet cell into specific hormone-producing phenotypes. Promoter regions involved in the regulation of the transcription of the three genes interact with a family of similar proteins related to the homeodomain helix-turn-helix proteins involved in tissue differentiation. By interacting with these DNA control elements, these proteins collectively suppress or stimulate transcription of the genes. In addition, Dr. Habener has identified cAMP response elements (CREs) in the promoters of these three islet hormone genes. The CREs mediate cAMP-induced gene transcription and bind the bZIP proteins, so-called because their DNA-binding domains consist of a basic region involved in DNA site-specific recognition and a coiled-coil leucine "zipper" responsible for dimerization. The bZIP proteins include fos, jun, C/EBP, and particularly a subgroup of proteins, the CREB/ATFs, which are CRE-binding proteins related to the activating transcription factors whose transactivation properties are stimulated by viral gene-encoded products.

In his studies (supported in part by the National Institutes of Health) of the promoter that controls expression of the somatostatin gene in pancreatic islet cells, Dr. Habener has discovered SMS-UE, a DNA enhancer element. SMS-UE, a D cell–specific transcriptional regulator that acts synergistically with the CRE of the promoter, is a bipartite element with two interdependent functional domains, A and B. Domain A binds a CCAAT box–binding protein α-CBF, a transcription factor that also regulates expression of the human chorionic gonadotropin α-subunit gene. Domain B binds an islet cell–specific protein with characteristics similar to those of Isl-1, a transcriptional activator protein that binds to the E2 enhancer of the rat insulin-1 gene. SMS-UE also binds transcription factor CREB, but not CREM, the close homologue of CREB, on a site adjacent to or overlapping the 3' end of domain B. Dr. Habener showed that the carboxyl-terminal bZIP domain of CREB binds to the CRE of the somatostatin gene but is not sufficient for binding to SMS-UE. He also discovered evidence suggesting that CREB–SMS-UE binding requires stabilization by a region of the protein located within the transactivation domain.

Analyses of both mRNAs and genomic DNA fragments indicate a considerable complexity of CREB

and CREB-like products arising from alternative splicing of exons, creating tissue-type–specific mRNAs with deleted, inserted, and substituted exons. Dr. Habener has discovered a particularly high level of CREB gene expression in the Sertoli and germ cells of the testis (in work supported in part by the National Institutes of Health). This expression is regulated cyclically in the Sertoli cells, waxing and waning every 12 days in the seminiferous tubules. The rise and fall coincides with the splicing out or in, respectively, of an exon containing multiple stop codons, resulting in the translation of a truncated CREB lacking a nuclear translocation signal and the DNA-binding domain. Examination of the promoter region of the CREB gene revealed numerous control elements, including three CREs, indicating that the transcriptional expression of this gene is likely under autopositive feedback control. Dr. Habener postulates that the increase in expression of the CREB gene in the Sertoli cells results from the increased intracellular cAMP levels stimulated by the cyclical rise in the pituitary gonadotropin (follicle-stimulating hormone) that interacts with receptors known to exist on the Sertoli cells. The fall in expression is a consequence of the splicing in of the exon, resulting in a CREB that can no longer enter the nucleus and autoactivate the CREB gene.

In addition, Dr. Habener has discovered a second gene that encodes the protein CREM, which also comprises many exons, several of which are nearly identical to those of CREB. Pre-mRNAs transcribed from both the CREB and CREM genes undergo complex patterns of alternative splicing of exons in a highly tissue-specific manner. The goal is to understand the functional consequences of these alternatively spliced mRNAs and how they alter the functions of the CREB and CREM proteins.

The transcriptional activation of the angiotensinogen gene in liver cells in response to acute-phase reactants is mediated by a complex interplay of DNA-binding proteins, including glucocorticoid receptors, nuclear factor κB (NF-κB), and C/EBP (CCAAT/enhancer-binding protein) or C/EBP-related protein(s). Studies indicate that in basal (unstimulated) conditions in hepatocytes, constitutively expressed C/EBP binds the acute-phase response element (APRE), flanked on both sides by glucocorticoid response elements (GREs), providing a modest glucocorticoid-inducible transcription. However, upon the activation of the acute-phase response (for example, by tissue injury, foreign antigens, or pyrogenic toxins), the resultant cytokines that are produced (such as interleukin-1

[IL-1] and tumor necrosis factor [TNF]) activate hepatic receptors coupled to both cAMP/protein kinase A and phospholipid/protein kinase C pathways. These pathways lead to the translocation of NF-κB from the cytoplasm to the nucleus, where it competitively displaces C/EBP for binding to the APRE. Inasmuch as NF-κB is a more potent transactivator than C/EBP, the rate of angiotensinogen gene transcription is markedly elevated.

Dr. Habener has identified a novel nuclear protein that serves as a dominant negative inhibitor of transcription factors C/EBP and LAP (liver-activating protein). A ^{32}P-labeled LAP DNA-binding and dimerization domain zipper probe was used to isolate a clone that encodes CHOP10, a new C/EBP homologous protein. CHOP10 has strong sequence similarity to C/EBP-like proteins within the bZIP region corresponding to the DNA-binding domain consisting of a leucine zipper and a basic region. Notably, however, CHOP10 contains two prolines substituting for two residues in the basic region, critical for binding to DNA. Thus heterodimers of CHOP10 and C/EBP-like proteins are unable to bind their cognate DNA enhancer element. CHOP10 mRNA is expressed in many different rat tissues. Antisera raised against CHOP10 recognize a nuclear protein with an apparent molecular mass of 29 kDa. CHOP10 is induced upon differentiation of 3T3-L1 fibroblasts to adipocytes, and cytokine-induced dedifferentiation of adipocytes is preceded by the loss of nuclear CHOP10.

Co-immunoprecipitation of CHOP10 and LAP from transfected COS-1 cells demonstrated a direct interaction between the two proteins *in vivo*. Consistent with the structure of its defective basic region, bacterially expressed CHOP10 inhibits the DNA-binding activity of C/EBP and LAP by forming heterodimers that cannot bind DNA. In transfected HepG2 cells, expression of CHOP10 attenuates activation of C/EBP- and LAP-driven promoters. Dr. Habener proposes that CHOP10 is a negative modulator of the activity of C/EBP-like proteins in certain terminally differentiated cells; this is similar to the regulatory function of Id on the activity of MyoD and MyoD-related proteins important in the development of muscle cells.

During the next year research will be focused on more-detailed investigations of the functional domains of the cAMP-responsive DNA-binding proteins. Studies will be aimed at defining the DNA-binding properties and the molecular processes of transcriptional activation. Particular emphasis will be on investigations of the roles of phosphorylations in nuclear transport, DNA binding, dimerization for-

mation of transactivation surfaces, and the coupling to other transcription factors. The structure and promoter region of the CREB gene, the diversity of additional CREB-like gene products in other tissues, and the structure and characteristics of the C/EBP-like proteins involved in the acute-phase response will be investigated. Efforts will also be made to isolate the cDNAs encoding the upstream and downstream DNA-binding proteins that cooperatively enhance cAMP-mediated activation of transcription of the glucagon and somatostatin genes.

Dr. Habener has also focused investigations on the cell-specific post-translational processing of proglucagon. Previously he determined the sequence of the rat glucagon gene and discovered that the gene encodes a prohormone that includes not only glucagon but two additional peptides related in structure to glucagon, the glucagon-like peptides. Marked differences in the pattern of post-translational processing of glucagon were found in rat pancreas and intestine. The intestine produces predominantly glucagon-like peptides, and the pancreas produces glucagon.

Having established that proglucagon encodes new glucagon-like peptides, Dr. Habener investigated the potential biologic activities of these peptides. Glucagon-like peptide-I (GLP-I) is a potent insulinotropic peptide. When studied in pancreatic islet cell lines, GLP-I(7–37) stimulates insulin gene transcription, cAMP formation, and insulin secretion at concentrations in the picomolar range. Moreover, GLP-I(7–37) stimulates insulin release in the perfused rat pancreas at concentrations as low as 10^{-12} M. Dr. Habener has determined that the pancreatic β-cell receptor for GLP-I(7–37) is distinct from that of glucagon in hepatic cells and that the receptor on β cells undergoes rapid homologous desensitization in a ligand-concentration manner. Efforts (supported in part by the National Institutes of Health) are being made to clone the β-cell receptor for GLP-I(7–37). Administration of the synthetic peptide to both nondiabetic and diabetic (type II) humans results in a marked increase of plasma insulin levels followed by a fall in blood glucose levels. Dr. Habener is testing the therapeutic properties of GLP-I in patients with type II (non-insulin-dependent) diabetes mellitus to determine whether the peptide can ameliorate the abnormal rise in blood sugar after meals and return fasting sugar levels toward normal levels.

Dr. Habener is also Professor of Medicine at Harvard Medical School and Associate Physician at Massachusetts General Hospital, Boston.

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CONTROL OF GENE EXPRESSION IN THE MAMMALIAN CEREBELLUM AND DURING THE CELL CYCLE

NATHANIEL HEINTZ, PH.D., *Investigator*

The studies in Dr. Heintz's laboratory focus on the identification of molecular mechanisms controlling gene expression in the developing cerebellum and during the cell cycle. The elucidation of these mechanisms should provide fundamental insights into the biological transitions that underlie the development of the mammalian central nervous system and the control of cell division.

Molecular Approaches to Understanding Development of the Mammalian Cerebellum

The mammalian cerebellum is a complex and highly stereotyped structure in which major pattern formation and functional organization occur postnatally. It is therefore amenable to study, and its development has been described in detail at the histological level. An extensive literature has documented the importance of cell-cell interactions in the generation and maintenance of normal cerebellar architecture. Furthermore, there are at least eight recessive mutations in inbred mouse strains that perturb cerebellar structure and function.

Dr. Heintz's laboratory has initiated several approaches toward identification of genes that are either required for—or respond to—specific transitions that occur during the development of the mammalian cerebellum. To identify genes important for the normal function of the cerebellum, the laboratory has initiated molecular genetic approaches to isolate several genes responsible for inherited neurologic disorders of the mouse. Significant progress has been made toward identifying the genes responsible for the *lurcher* (*Lc*) and *meandertail* (*mea*) mutant genes.

The well-studied semidominant *Lc* mutation results in degeneration and death of essentially all cerebellar Purkinje cells, commencing ~10 days after birth. Recent light and electron microscopic studies, combined with *in situ* hybridization experiments, have strongly suggested that *Lc* Purkinje cells die by the process of apoptosis. Identification of the

Lc mutation should provide fundamental insights into the molecular events that can ectopically activate programmed cell death in inherited neurodegenerative diseases. Genetic mapping studies have identified two molecular markers that flank the *Lc* gene by ~0.5 cM. One marker has been used to screen a yeast artificial chromosome (YAC) mouse genomic library prepared by Dr. Shirley Tilghman's laboratory (HHMI, Princeton University) to identify a 280-kb YAC clone containing mouse genomic DNA near the *Lc* locus. This resource and correlation of the physical and genetic maps will allow an oriented genomic walk toward the *Lc* gene.

A similar approach is under way to isolate the *mea* gene, an intriguing recessive mutation that results in skeletal abnormalities in the tail and cerebellar abnormalities. In collaboration with Drs. Mary Beth Hatten and Carol Mason (Columbia University College of Physicians and Surgeons), Dr. Heintz's laboratory has discovered that the *mea* gene influences compartmental organization in the mammalian brain. Three-dimensional reconstructions of the *mea* cerebellum have established that this mutation results in deletion of a specific quadrant of the mouse cerebellum. A molecular probe mapping ~0.5 cM from the *mea* gene has been identified. Genetic analysis of two additional neurologic mutants, *tottering* (*tg*) and *nervous* (*nr*), is also under way. The eventual cloning of these and other genes identified by mouse neurological mutations will provide important insights into the development of the mammalian brain.

A variety of subtractive cloning techniques have also been employed to isolate a large number of cDNA clones for mRNAs that are cell specific or developmentally regulated during cerebellar development. These clones are now being analyzed to decide which are specific markers for developmental transitions that occur during the formation of the cerebellum. During the past year, *in situ* hybridization studies have established that specific stages in

the development of cerebellar neurons are marked by expression of these genes. Dr. Heintz and his colleagues are interested in identifying the molecular mechanisms that regulate such stage-specific expression and in understanding the functions of these novel developmentally regulated proteins in the mammalian cerebellum.

Control of Macromolecular Synthesis During the Cell Cycle

In studies supported principally by the National Institutes of Health, Dr. Heintz's laboratory has established that transcriptional induction of histone gene expression during the S phase of the mammalian cell cycle involves coordinate activation of a set of transcription factors that interact with subtype-specific consensus sequences within the histone gene promoters. Thus each of the approximately dozen genes encoding histone H2b contains a highly conserved element that interacts with the transcription factor Oct-1 to promote S-phase–specific transcription. Distinct but functionally similar subtype-specific consensus elements and their cognate transcription factors are also important for cell cycle–control of histone H1 and H4 genes. The transcription factors (Oct-1, H1TF1, H1TF2, and H4TF2) interacting with these cell cycle–control sequences have been isolated and characterized, and their behavior during the cell cycle is now being investigated.

Recent investigations have established that the H2b transcription factor Oct-1 undergoes a complex program of phosphorylation during the cell cycle that correlates with changes in its functional activity. One aspect of this program is the mitotic hyperphosphorylation of Oct-1, which leads to its functional inactivation at this time in the cell cycle. Thus phosphorylation within the Oct-1 DNA-binding domain occurs *in vivo* and can be reproduced *in vitro* using purified protein kinase A (PKA). These observations demonstrate that post-translational modifications play an important role in regulating transcription factor activity during mitosis, and implicate PKA in the regulation of transcription by the POU family of DNA-binding proteins.

Present efforts are directed toward identifying both the enzymes responsible for additional modifications of Oct-1 during the cell cycle and their functional consequences. To determine whether post-translational modifications also modulate the activity of other histone gene transcription factors implicated in cell cycle control, the laboratory has extended these studies to H1TF2, a transcription factor that binds to the histone H1 S-phase regulatory sequence. This factor is a novel heterodimeric complex that is entirely distinct biochemically from Oct-1, yet the results indicate that it is also regulated late in the cell cycle by phosphorylation. This provides the first biochemical evidence that coordinate control of histone gene transcription during the cell cycle appears to be effected through molecular mechanisms that modulate functionally related but biochemically distinct factors.

Of particular interest is the possibility that either these transcription factors or the mechanisms regulating their activity might also be utilized to control the initiation of DNA synthesis during S phase. In collaboration with Dr. Lisa Dailey (Rockefeller University) and Dr. Nicholas Heintz (University of Vermont), Dr. Heintz and his colleagues have recently demonstrated that Oct-1 interacts with the dihydrofolate reductase (*dhfr*) origin of DNA replication and have identified novel activities (RIP60, RIP100) that have properties expected of bona fide mammalian DNA replication-initiation factors. Thus RIP60 is a sequence-specific DNA-binding protein that interacts adjacent to an AT-rich, bent DNA segment in the *dhfr* origin. It also binds specifically to two sites within the yeast ARS1 (autonomously replicating sequence) origin of replication that have been shown to be essential for efficient ARS1 function. RIP100 is an ~100-kDa ATP-binding protein that copurifies with significant DNA helicase activity and interacts with RIP60. These and other features of the purified RIP60/RIP100 preparations suggest that they may be involved in chromosomal DNA synthesis. Further efforts are directed toward definitive demonstration that this is the case and investigations into possible mechanisms for coordinate regulation of transcription and DNA replication during the S phase of the mammalian cell cycle.

Dr. Heintz is also Professor of Molecular Biology at the Rockefeller University.

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MOLECULAR ANALYSES OF CELL-MATRIX ADHESION

RICHARD O. HYNES, PH.D., *Investigator*

Dr. Hynes and his colleagues are involved in molecular and cellular analyses of cell adhesion and its role in a variety of physiological processes, including embryological development, hemostasis, thrombosis, wound healing, and cancer. The research is concentrated on a set of adhesive extracellular matrix proteins known as fibronectins and on a set of cell surface receptors known as integrins.

Fibronectins comprise a set of related but different proteins, all derived from a single gene by alternative splicing of the initial 70-kb transcript to give multiple mRNAs of 8–9 kb. These mRNAs differ by inclusion or exclusion of three segments and therefore encode slightly different proteins. One area of research concerns the molecular basis and the physiological consequences of the alternative splicing of fibronectins. The alternatively spliced exons are differentially expressed in different cells and tissues, and the pattern of splicing is altered during development and in response to physiological stimuli. Two of the segments (A and B) are always present in the fibronectin associated with cell migration during development but are selectively excluded by various cell types later in development; e.g., both are excluded from fibronectin mRNA in adult skin. However, after wounding of the skin there is a marked increase in the levels of fibronectin mRNA, and this mRNA contains both A and B segments, as in embryos. This suggests that A^+B^+ fibronectin may be important for the migration and/or proliferation that occurs both in developing embryos and in healing wounds.

To test this and other hypotheses based on studies of the patterns of expression of different fibronectin isoforms, Dr. Hynes and his colleagues constructed recombinant fibronectin genes and introduced them into cells. Cell lines were produced that secrete in pure, homogeneous form each of the types of fibronectin that, in nature, are found in mixtures. In this way it is possible to purify the different types in quantity and to assay their biological functions. One result arising from these studies is that certain lymphoid cells adhere specifically only to those forms of fibronectin containing the third alternatively spliced segment (V). The binding site within the V region was mapped to a 10–amino acid stretch. These results define an alternatively spliced cell-type–specific cell adhesion site in fibronectin. The integrin receptor ($\alpha_4\beta_1$) recognizing this site was identified by affinity chromatography on synthetic peptides and by specific antibody blocking.

Similar studies are under way to analyze further the roles of the other alternatively spliced segments in the behavior of various cell types.

To investigate the roles of different fibronectin isoforms *in vivo*, Dr. Hynes and his colleagues are altering the expression of fibronectins in strains of mice. Homologous recombination in embryonic stem (ES) cells is being used to "knock out" the fibronectin gene or to mutate it to alter its pattern of splicing. The altered ES cells have been reintroduced into embryos, and mutant strains of mice have been derived. These strains reveal that fibronectin is essential for early embryonic development, since the embryos die early in development. Further analyses are under way to elucidate the exact defects produced by elimination or alterations of the fibronectin gene. Transgenic mice expressing specific isoforms of fibronectin have also been generated and are currently being analyzed. Interbreeding of these various strains of mice will allow detailed genetic analyses of the roles of the different forms in development and in various physiological and pathological processes.

The second major area of interest in the laboratory concerns integrins, transmembrane receptors made up of α and β subunits. There are at least 8 β subunits and at least 12 α subunits. Different $\alpha\beta$ combinations generate receptors with different but overlapping specificities for various adhesive extracellular matrix proteins, including fibronectins. The α and β subunits interact, via their large extracellular domains, with these adhesive proteins and, via their small cytoplasmic domains, with cytoskeletal proteins. Thus they serve to link the extracellular matrix to the cytoskeleton. The spectra of integrins expressed by different cells vary and alter during development and in response to various stimuli. For example, oncogenically transformed cells lose certain integrins; this loss probably contributes to their altered ability to adhere to and assemble extracellular matrices and thus to their aberrant behavior. This model is under test by transfection experiments, in which integrin genes are introduced into cells and their properties analyzed.

The roles of several integrins *in vivo* are being studied by homologous recombination in murine ES cells and generation of mutant mice, as described above for fibronectins. The roles of integrins in development are also being studied genetically in the fruit fly *Drosophila*. The genes for several *Drosophila* integrin subunits have been cloned, and mutants

are available for some. Dr. Hynes and his colleagues have identified alternatively spliced forms of integrins, as well as novel integrins in *Drosophila*, and are analyzing their expression and function. Using the available mutations, the laboratory has demonstrated roles for certain integrins in the development of early embryos, muscles, wings, and eyes. By using P-element–mediated transformation to introduce integrin genes into the fly genome, the mutant phenotypes can be rescued, and, by appropriate constructions, the times at which integrins are required and the roles of the different spliced forms have been investigated. Site-specific mutagenesis is being used to analyze the roles of specific segments of the integrin subunits. In particular, the roles of the cytoplasmic domains thought to be involved in cytoskeletal connections are under study.

The functions of the cytoplasmic domains are also being examined by transfection of mutated integrin subunit genes into mammalian cells. These experiments implicate the cytoplasmic domain of the β_1 subunit in association with the cytoskeleton. Similar studies are being initiated on the α subunits, particularly three related integrins ($\alpha_3\beta_1$, $\alpha_4\beta_1$, $\alpha_5\beta_1$) that are all receptors for fibronectin, albeit for different regions, including, for $\alpha_4\beta_1$, one of the alternatively spliced segments.

In parallel with these studies on integrins, talin, one of the cytoskeletal proteins thought to interact with integrins, has been cloned and sequenced. This has allowed definition of its overall structure and the homology of one domain with other membrane-associated cytoskeletal proteins. These clones are being used to determine the binding sites for potential interacting proteins, including integrins and vinculin, another cytoskeletal protein involved in connections between integrins and actin. The aim is to elucidate the molecular structure of the "focal"

contact, where extracellular matrix is connected to the cytoskeleton via integrins. Focal contact organization and function are altered in tumor cells. Thus it is an important point of regulation of cell behavior.

The work on *in vitro* expression of integrins and fibronectins and some of the research on mice is supported by grants from the National Cancer Institute and the National Heart, Lung and Blood Institute.

Dr. Hynes is also Professor of Biology at the Massachusetts Institute of Technology.

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FACTORS THAT PROMOTE HOST CELL ENTRY AND GROWTH OF INTRACELLULAR BACTERIA

RALPH R. ISBERG, PH.D., *Assistant Investigator*

Dr. Isberg and his colleagues are investigating the interaction of intracellular bacterial pathogens with human cells. The main concern of the laboratory is to determine how some pathogenic microorganisms are able to penetrate into host cells that are not normally considered phagocytic and how bacteria are able to evade intracellular killing mechanisms and thrive within host cells. To dissect these processes, Dr.

Isberg's laboratory has concentrated on two gram-negative bacteria that are either efficient at entry into host cells or grow well within the normally hostile environment of phagocytes. Bacterial uptake into cultured mammalian cells is being studied with the bacterium *Yersinia pseudotuberculosis*, while *Legionella pneumophila* is being studied as a model for intracellular growth within macrophages.

Growth of *Legionella pneumophila* in Cultured Phagocytes

Dr. Isberg's laboratory has been analyzing the growth of *L. pneumophila* in monocytic cells, which normally kill invading microorganisms. *L. pneumophila*, the causative agent of Legionnaire's disease pneumonia, initiates infection in human hosts by growing within lung macrophages. This process can be reproduced in cultured cell models, which indicates that this microorganism has a unique intracellular life style. Most bacteria internalized by macrophages are found in a phagosome that fuses with a lysosomal compartment, so that the microorganism is exposed to a variety of toxic factors. *L. pneumophila*, on the other hand, alters normal organelle traffic in the host cell, so that the phagosome does not fuse with lysosomes, allowing the bacterium to occupy a protected niche called the replicative phagosome. Once within this replicative phagosome, recruitment of host organelles to this site occurs, as mitochondria, ribosomes, and smooth vesicles are found surrounding this compartment. The bacterium then grows in this site. The nature of these various trafficking events is being analyzed, and genetic approaches have been devised to determine the relative importance of these morphological changes in supporting intracellular growth of *L. pneumophila*.

To analyze the nature of the organelle trafficking events that occur in the macrophage after an *L. pneumophila* infection, Dr. Michele Swanson has identified host cell markers that colocalize with the replicative phagosome. The results from these studies indicate that the phagosome takes part in fusion with compartments that are part of the early stages of the macrophage secretory pathway. Surprisingly, she finds that growth of the microorganism occurs within the host endoplasmic reticulum. Recent studies on a variety of other intracellular bacteria and viruses have indicated that this may be a common replication site and that there may be a poorly characterized pathway of fusion events that allows delivery of endocytosed material to the endoplasmic reticulum. Current efforts that use a variety of mutant strains are devoted to identifying bacterial factors encoded by the bacterium that route the organism to this site.

To analyze how *L. pneumophila* causes drastic alterations in organelle trafficking, Karen Berger has isolated bacterial mutations. The mutants fall into three phenotypic classes. Class I mutants enter via the normal receptor pathway used by the wild type, they inhibit phagolysosome fusion normally, but they are unable to fuse with the endoplasmic reticulum. Class II mutants, which are the most common, are defective for inhibiting phagolysosome fusion. Class III mutants, unlike the other classes, are only slightly defective for growth, are unaffected for fusion with the endoplasmic reticulum, but appear to be engulfed by both the normal route of receptor uptake as well as a second pathway for uptake. To characterize these mutants further, the laboratory isolated molecular clones that could genetically complement the defects in class I and class II mutants. A single 7-kb region of the *L. pneumophila* chromosome could complement these two classes, even though the phenotypes of these mutants are different. The mutations from both classes appear to lie in a single gene, now called *dot* (for defective organelle trafficking), which encodes a 112-kDa hydrophobic protein. Mutations that cause a defect in inhibiting phagolysosome fusion appear to make a totally defective Dot protein. Mutants that are able to inhibit phagolysosome fusion but are unable to cause organelle recruitment, on the other hand, seem to have partial Dot activity. Numerous strategies are currently being pursued to identify the biochemical activity of the Dot protein that affects these processes.

Susannah Rankin developed a final approach toward analyzing intracellular growth of *L. pneumophila*, in which genetic loci are identified that are expressed exclusively when the organism is growing in an intracellular environment but that are turned off when the organism is growing extracellularly. To this end, a clone bank was constructed in a vector harboring a promoterless gene encoding a protein involved in thymidine biosynthesis. The bank was then introduced into an auxotroph requiring expression of this gene to grow intracellularly. Clones that survived this enrichment were then grown extracellularly under conditions in which expression of this gene is lethal. Strains that survived both enrichments contained molecular clones with loci that are expressed exclusively when the organism is growing intracellularly. With this strategy at least eight genes were cloned that are normally only expressed by *L. pneumophila* when the microorganism is within a host cell.

Yersinia pseudotuberculosis Entry Into Mammalian Cells

To investigate the entry of *Y. pseudotuberculosis* into cultured mammalian cells, members of Dr. Isberg's group have been studying invasin, a 986–amino acid outer membrane protein encoded by this microorganism. Previous work from the laboratory had shown that the carboxyl terminus of invasin binds to four members of the β_1-chain integrin family of cell adhesion molecules, which are heterodi-

meric receptors found on a wide variety of mammalian cells. Analysis of this process has involved studying mutations located both in invasin and its integrin receptors that affect binding or uptake of bacteria.

Dr. Guy Tran Van Nhieu in the laboratory has analyzed the relationship between the binding of a ligand to an integrin and bacterial uptake into host cells. He has shown that high-affinity binding is a critical determinant for bacterial uptake into mammalian cells and that if there is insufficient affinity, the bacteria will simply adhere to the integrin without being internalized by the mammalian cell. To analyze the role of the integrin receptor in uptake, he has been analyzing mutations located in the β_1-integrin chain, some of which were provided by Dr. Alan F. Horwitz (University of Illinois). There are two surprising results from these experiments. First, although uptake of the bacterium requires rearrangement of the host cell cytoskeleton, the integrin is probably not binding the cytoskeleton during the uptake process. Second, factors involved in receptor-mediated endocytosis are probably important for phagocytosis of microorganisms, and these factors must interact with the integrin during the bacterial uptake process. Studies are in progress to identify these factors that interact with the integrin

receptor. The work on uptake of *Y. pseudotuberculosis* was supported by a grant from the National Institute of Allergy and Infectious Diseases, National Institutes of Health.

Dr. Isberg is also Associate Professor of Microbiology and Molecular Genetics at Tufts University School of Medicine, Boston.

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FUNCTIONAL INTERACTIONS BETWEEN INTRAMEMBRANE CHARGED RESIDUES IN THE LACTOSE PERMEASE OF *ESCHERICHIA COLI*

H. Ronald Kaback, M.D., *Investigator*

The lactose (lac) permease of *Escherichia coli* is a hydrophobic, polytopic cytoplasmic membrane protein that catalyzes the coupled translocation of β-galactosides and H^+ with a 1:1 stoichiometry (i.e., symport or cotransport). As such, this protein is a paradigm for a large number of secondary transporters that catalyze cation-coupled translocation reactions in diverse membranes ranging from those of archaebacteria to the mammalian central nervous system. Encoded by the *lacY* gene, the permease has been solubilized from the membrane, purified, reconstituted into proteoliposomes, and shown to be solely responsible for β-galactoside transport, probably as a monomer. Based on circular dichroic studies and hydropathy analysis of the deduced amino acid sequence, a secondary structure was proposed in which the permease has a short hydrophilic amino terminus, 12 hydrophobic domains in α-helical configuration that traverse the membrane in zigzag fash-

ion connected by hydrophilic loops, and a 17-residue hydrophilic carboxyl-terminal tail. Evidence supporting the general features of the model and demonstrating that both the amino and carboxyl termini are on the cytoplasmic face of the membrane has been obtained from other spectroscopic techniques, limited proteolysis, immunological studies, and chemical modification. Analysis of an extensive series of *lac* permease–alkaline phosphatase (*lacY-phoA*) fusions has provided exclusive support for the 12-helix motif.

Recently it was found that lac permease mutants with Thr in place of Lys358 or Asn in place of Asp237 are defective with respect to active transport. Second-site suppressor mutations of K358T yield neutral amino acid substitutions for Asp237 (Asn, Gly, or Tyr), while suppressors of D237N exhibit Gln in place of Lys358. Based on these findings, it was proposed that Asp237 and Lys358 inter-

act via a salt bridge, thereby neutralizing each other. Replacement of either charged residue with a neutral residue creates an unpaired charge that causes a functional defect, while neutral substitutions for both residues do not cause inactivation. Consequently, the secondary-structure model proposed for the permease was altered to accommodate a putative salt bridge between Asp237 and Lys358 in the low dielectric of the membrane, by placing residues Phe247 to Thr235 in transmembrane helix VII rather than in the hydrophilic domain between helices VII and VIII.

As part of an extensive site-directed mutagenesis study with an engineered lac permease devoid of Cys residues (C-less permease), putative intramembrane residues Asp237, Asp240, Glu269, Arg302, Lys319, His322, Glu325, and Lys358 were systematically replaced with Cys; individual replacement of any of the residues essentially abolishes active lactose transport. The single Cys mutants D237C and K358C were used to construct a double mutant containing both Cys substitutions in the same molecule. D237C/K358C transports lactose at about half the rate of C-less permease to almost the same steady-state level of accumulation. Moreover, replacement of Asp237 and Lys358, respectively, with Ala and Cys or Cys and Ala, or even interchanging Asp237 with Lys358, causes little change in activity. These observations provide a strong indication that Asp237 and Lys358 interact to form a salt bridge and that neither residue nor the salt bridge is important for transport. Despite the relatively high activity of the charge-inversion mutant (D237K/K358D) and the mutants with neutral substitutions at positions 237 and 358, immunoblots reveal low levels of the polypeptides in the membrane, suggesting a role for the salt bridge in permease folding and/or stability. The observations also suggest that Asp237 and Lys358 may interact in a folding intermediate but not in the mature molecule. Remarkably, however, an inactive mutant with Cys in place of Asp237 regains full activity upon carboxymethylation, which restores a negative charge at position 237. Therefore it seems likely that the interaction between Asp237 and Lys358 is important for folding and/or stability and that the residues maintain proximity in the mature permease.

To test the possibility that other charged residues in transmembrane helices are neutralized by charge-pairing, Dr. Kaback and his co-workers constructed 13 additional double mutants in which all possible interhelical combinations of negative and positively charged residues were replaced pairwise with Cys. Only the double mutant D240C/K319C exhibits significant transport activity. However, the interaction

between Asp240 and Lys319 is different phenomenologically from that of Asp237-Lys358. Thus D240C/K319C catalyzes lactose transport at about half the rate of C-less permease to a steady-state level that is only ~25–30% of the control. Moreover, although significant activity is observed with the double-Ala mutant or with the two possible Ala-Cys combinations, interchanging Asp240 and Lys319 completely abolishes active transport. Therefore, although neither Asp240 nor Lys319 *per se* is mandatory for active transport, the polarity of the interaction is apparently important for full activity. Finally, unlike the double mutants in D237 and K358, all of the D240/K319 mutants are found in the membrane in amounts comparable to the C-less control.

The results suggest that charge-pairing between intramembrane charged residues is probably not a general feature of lac permease and may be exclusive to D237/K358 and D240/K319. However, the charge-pair neutralization approach depends on permease activity and will not reveal charge-paired residues if they are essential for activity. Double-Cys mutants involving residues suggested to be H-bonded and directly involved in lactose-coupled H^+ translocation and/or substrate recognition (i.e., Arg302, His322, and Glu325, as well as Glu269, which has been shown to be essential) are inactive with respect to active lactose transport.

The modified secondary-structure model of lac permease is based on the functional interaction between Asp237 and Lys358 and on the notion that the intramembrane charged residues are balanced. Despite the indication that Asp240 and Lys319 may also participate in a salt bridge, the evidence for the interactions is indirect; other approaches are required to determine the location of the residues relative to the plane of the membrane and to demonstrate directly that the pairs are in close proximity. C-less permease mutants containing double-Cys or paired Cys-Ala replacements will be particularly useful. Preliminary efforts to estimate the accessibility of Cys residues at positions 237 and 358 with water- or lipid-soluble sulfhydryl reagents suggest that Cys residues at the two positions are accessible to both types of reagents, although the lipid-soluble reagents are more effective. Other experiments with permease mutants specifically labeled with paramagnetic or fluorescent probes, as well as a series of alkaline phosphatase fusions in helix VII, are consistent with placement of both residues near the interface at the external surface of the membrane rather than in the middle of helix VII. Efforts to demonstrate disulfide bond formation directly by oxidation of appropriate double-Cys mutants are also in progress. Based on the current evidence, it is clear

that Asp237-Lys358 and Asp240-Lys319 interact functionally, and it is reasonable to suggest that both pairs of residues may be in close proximity. Thus putative helix VII (Asp237 and Asp240) may neighbor helices X (Lys319) and XI (Lys358) in the tertiary structure of the permease.

Dr. Kaback is also Professor of Physiology and of Microbiology and Molecular Genetics and a member of the Molecular Biology Institute at the University of California, Los Angeles.

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PROTEIN FOLDING AND MACROMOLECULAR RECOGNITION

Peter S. Kim, Ph.D., *Assistant Investigator*

Dr. Kim and his colleagues are interested in understanding protein folding and macromolecular recognition. The laboratory is also using peptide and protein design to test and improve the knowledge base in folding and recognition.

Protein Folding

The mechanism of information transfer from one to three dimensions is a major unsolved problem in molecular biology. Much of the laboratory's work in protein folding is centered on bovine pancreatic trypsin inhibitor (BPTI), arguably the most thoroughly characterized protein in biophysical terms. The native structure of BPTI is stabilized by three disulfide bonds. Following the pioneering studies of Dr. Thomas E. Creighton (European Molecular Biology Laboratory), Dr. Kim and his colleagues have determined the oxidative folding pathway for BPTI. Recent work has focused on characterization of early stages in this pathway and on determining the folding pathway for pro-BPTI.

Three approaches are being used to obtain structural information about BPTI-folding intermediates. First, peptide models for the early folding intermediates are designed and studied. These models indicate that subdomains of native structure are stable in isolation. Second, recombinant models for the disulfide-bonded intermediates are made by replacing some of the cysteine residues with alanine. One variant containing only a single disulfide bond is folded completely into a native BPTI structure and offers a new system for studies of protein folding. Third, the dynamic properties of BPTI variants containing a subset of disulfide bonds are being studied by mea-

suring individual amide-proton exchange rates in two-dimensional nuclear magnetic resonance experiments. Initial results indicate that there can be significant differences in the dynamic properties of BPTI variants, even when the overall structure is the same. This research was supported by a grant from the National Institutes of Health.

A large part of the protein-folding problem is the identification and characterization of subdomains of native proteins. Accordingly, protein architecture and protein structure hierarchies are being analyzed and algorithms developed to predict autonomously folding domains and subdomains of proteins. In addition, proteins are "dissected" experimentally to identify minimal folding units and to test predictions of these algorithms. Protein fragments that correspond to minimal folding units have been identified and are being characterized. In other protein-folding efforts, electrostatic interactions at the termini of α helices are being studied in isolated peptides. The properties of β sheets are being investigated in a small β-sheet–rich domain of protein G, the Fc receptor from *Streptococcus*.

Macromolecular Recognition

A second focus of Dr. Kim's laboratory is in macromolecular recognition, which is central to much of molecular physiology. The laboratory is particularly interested in the leucine zipper class of DNA-binding proteins, originally identified by Dr. Steven McKnight (HHMI, Carnegie Institution) and his co-workers. The leucine zipper regions of these proteins are important for homodimer or specific heterodimer formation. The long-term goal is to understand the "code" for specificity and stability of leucine zipper interactions.

GCN4, a homodimeric transcription factor, serves as a prototype protein. A synthetic peptide corresponding to the 33-residue leucine zipper region of GCN4 has been shown to be a two-stranded parallel coiled coil. X-ray crystallographic studies of this peptide (in collaboration with Dr. Tom Alber, University of California, Berkeley) provide the first high-resolution structure of this motif in isolation. The effects of amino acid replacements and/or deletions on the stability, structure, dynamics, and oligomerization of this leucine zipper, as well as the effects on DNA binding in the context of a larger domain, are being investigated.

The isolated leucine zipper regions from the nuclear oncogene products Fos and Jun are sufficient to mediate specific heterodimer formation. The Fos homodimer contains several acidic residues that lead to substantial electrostatic repulsion at neutral pH. It is the destabilization of the Fos homodimer

that provides a major thermodynamic driving force for heterodimer formation. (This research was supported by a grant from the National Institutes of Health.)

Peptide and Protein Design

Knowledge of the rules involved in protein folding and macromolecular recognition can be tested by trying to design amino acid sequences that fold into specific conformations and/or interact in a predetermined manner with other molecules. Based on studies of the mechanism of heterodimer formation between the Fos and Jun leucine zippers described above, two peptides with Velcro-like properties have been designed; each by itself is predominantly unfolded in aqueous solution, but when mixed they form a stable heterodimeric coiled coil.

Efforts to modify the hydrophobic interface of the GCN4 homodimeric coiled coil led to surprises. Simply changing the nature of the hydrophobic residues at the 4–3 hydrophobic repeat position (i.e., while keeping all other residues the same) can change the oligomerization state of the peptides from dimer to trimer to tetramer. The structure of the tetramer has been solved by x-ray crystallography (in collaboration with Dr. Alber). This structure corresponds to a new structural motif, the parallel four-helix coiled coil, containing a small channel coincident with the superhelix axis.

Other protein design efforts include alteration of the surface of a small water-soluble protein without changing the hydrophobic core, in an attempt to design a model intrinsic membrane protein.

Dr. Kim is also Member of the Whitehead Institute for Biomedical Research, Associate Professor of Biology at Massachusetts Institute of Technology, and Assistant Molecular Biologist at Massachusetts General Hospital, Boston.

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GENETICS AND BIOCHEMISTRY OF RNA VIRUSES

KARLA A. KIRKEGAARD, PH.D., *Assistant Investigator*

RNA virus and virus-like genomes replicate in cells of bacteria, fungi, plants, and animals. Many proteins and subcellular structures from the host cells are undoubtedly used by the viruses during their replicative cycles, but only a few of these host components have been identified. For example, $Q\beta$ phage utilizes translation factors from its *Escherichia coli* host as subunits of its RNA replicase, encoding only the catalytic subunit with its own genome. Poliovirus, a positive-strand RNA virus, can specifically infect primate cells because the poliovirus receptor, a cellular adhesion protein identified in the laboratory of Dr. Vincent Racaniello (Columbia University), is present on surfaces of these host cells. During the 7-h course of a lytic infection with poliovirus, numerous changes occur in the host cells: cellular translation is inhibited, transcription is depressed, and membranous vesicles with which the viral replication machinery is specifically associated accumulate. Dr. Kirkegaard's laboratory is interested in identifying proteins in mammalian cells employed during poliovirus infection by assaying their altered function during infection, their specific binding to poliovirus proteins, and any changes in these properties during infection with well-characterized mutant polioviruses.

The budding yeast *Saccharomyces cerevisiae*, in which virus-like particles containing double-stranded RNA genomes replicate in large numbers, provides a system in which the contributions of host proteins to RNA viral replication can be assessed genetically. As many as 100,000 copies per cell of one such virus, L-A, are present in most laboratory strains of *S. cerevisiae*. Unlike lytic viruses, the presence of L-A particles is apparently not detrimental to yeast cells. Dr. Kirkegaard's laboratory has found that under certain conditions the L-A virus particles provide a growth advantage to its host, suggesting a mutualistic relationship between the RNA genome and the yeast cell.

Inhibition of Protein Secretion in Host Cells During Poliovirus Infection

The origin of the membranous vesicles that accumulate during poliovirus infection of primate cells and the mechanism of their formation are unknown. However, recent findings from the Kirkegaard laboratory argue that these vesicles are derived from the normal protein secretion apparatus of the infected cell, either from the endoplasmic reticulum (ER) or the cis compartment of the Golgi apparatus. First, the surprising observation that brefeldin A, an inhibitor of ER-to-Golgi transport, specifically inhibited poliovirus RNA synthesis suggested that poliovirus RNA replication requires normal ER and Golgi function. However, poliovirus encodes no glycosylated or secreted proteins; it is thus possible that the function required from the host ER or Golgi might be the vesicle formation through which normal protein traffic occurs.

Two recent sets of experiments have supported this model. In brefeldin A–resistant cell lines, poliovirus replication was found to be brefeldin A resistant; therefore it is a cellular function that mediates the inhibition observed in wild-type cells. Furthermore, when VSV G, a membrane protein used to follow the glycosylation events that accompany normal protein secretion, was expressed in poliovirus-infected cells, Dr. Kirkegaard and her colleagues found that its transit through the protein secretion apparatus halted between the ER and the medial Golgi compartment. Currently they are testing whether the vesicles that accumulate during poliovirus infection bear ER or cis-Golgi markers and if

any viral proteins can independently accomplish this inhibition. *In vitro* protein secretion assays may reveal the identity of the host components whose altered function during poliovirus infection inhibits protein secretion. This project was also supported by a grant from the National Institutes of Health.

Recombination and Replication of Polioviral RNA

One mechanism by which such a wide variety of RNA viruses is generated is thought to be genetic recombination. In poliovirus, recombination between RNA genomes occurs via the switching of the viral RNA replication complex between parental templates. The Kirkegaard laboratory has developed a polymerase chain reaction (PCR) assay for RNA recombination to detect and quantify the formation of recombinant molecules at frequencies as low as 1 in 10^6. A collection of temperature-sensitive polymerase mutants generated by charged-cluster-to-alanine scanning mutagenesis is currently being screened with this quantitative assay for any changes in recombination frequency. With the PCR assay it was found that, in contrast to previous reports, RNA recombination occurs throughout the infectious cycle of poliovirus, increasing in frequency late in infection. Thus the drastic reorganization of the cytoplasm during infection does not restrict the access of replicating RNAs to each other, and RNA recombination can occur whenever RNA polymerization occurs in the infected cell. This point is underscored by the increasing number of RNA viruses for which genetic recombination has been reported; the quantitative PCR assay developed in the Kirkegaard laboratory is suited for use in studying recombination frequencies between defined markers in any RNA genome.

Double-stranded RNA Viruses of *S. cerevisiae*

Three *S. cerevisiae* genes whose products are required for L-A maintenance have been identified in the laboratory of Dr. Reed Wickner (National Institutes of Health). One of these genes encodes an *N*-acetyltransferase that may stabilize the icosahedral capsid; the functions of the other two are unknown. A genetic screen in the Kirkegaard laboratory to identify more L-A maintenance mutants has yielded more than 100 candidate strains; complementation

analysis is in progress to determine the number of new loci identified.

To engineer mutations into the genome of an RNA virus and to study the effects of those mutations on the normal replicative cycle it is necessary to initiate the replicative cycle either with cloned DNA or with RNA molecules transcribed *in vitro* from cloned DNA. One goal of the Kirkegaard laboratory is to study the effects of defined mutations in the RNA genome of L-A on its replication in its *S. cerevisiae* host and, in turn, to be able to study the effects of defined host mutations on mutant and wild-type L-A replication. First, with RNA molecules encoding the firefly luciferase protein, methods to introduce RNA directly into yeast cells were developed. Then an L-A cDNA clone provided by Dr. Wickner was modified such that transcribed RNA nearly indistinguishable from single-stranded L-A RNA present in yeast cells could be synthesized. By using conditions under which yeast cells grow very poorly in the absence of the L-A virus, colonies of cells in which the L-A replicative cycle had been initiated by the direct introduction of RNA could be identified and characterized. Thus "infectious" L-A RNA can be synthesized from wild-type and, presumably, mutated cDNA clones, and the yeast RNA virus will now be accessible to direct genetic analysis.

Dr. Kirkegaard is also Assistant Professor of Molecular, Cellular, and Developmental Biology at the University of Colorado at Boulder and Adjunct Assistant Professor of Cellular and Structural Biology at the University of Colorado Health Sciences Center, Denver.

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MOLECULAR BASIS OF ADRENERGIC RECEPTOR FUNCTION

BRIAN K. KOBILKA, M.D., *Assistant Investigator*

Adrenergic receptors play an important role in the control of cardiovascular function by the central nervous system. The application of cloning, expression, and mutagenesis techniques has led to remarkable progress in the field of adrenergic receptor biology. Much has been learned about the functional domains responsible for ligand binding, G protein activation, and receptor desensitization. Research in Dr. Kobilka's laboratory addresses several areas of adrenergic receptor biology that remain poorly understood.

Receptor Structure

Dr. Kobilka and his colleagues have attempted to gain insight into the three-dimensional structure of adrenergic receptors by identifying intramolecular interactions within the receptor protein. Chimeric α_2/β_2 receptors were constructed to locate complementary mutations that restore functional properties to mutationally inactivated receptors. The effect of mutations on receptor function may result either from a direct modification of a functional domain or from a more general effect on the three-dimensional structure of the receptor. The structural alterations are likely to occur when the mutated domain is in close proximity to another domain of the receptor, particularly if the chemical or physical properties of the mutated amino acid are essential for maintaining the three-dimensional architecture of the protein.

Dr. Sankuratri Suryanarayana has taken advantage of these principles to study the structure of adrenergic receptors. When Asn^{312} of the β_2 receptor is mutated to Phe ($\beta_2 N \rightarrow F$), the amino acid found in the homologous location of the α_2 receptor, it is no longer functional and is retained in the endoplasmic reticulum. Inserting a sequence from the first and second hydrophobic domains of the α_2 receptor into $\beta_2 N \rightarrow F$ restores the ability to bind ligands; this chimeric receptor is localized to the plasma membrane. The converse mutation in the α_2 receptor ($\alpha_2 F \rightarrow N$) results in diminished agonist affinity. This abnormality can be complemented by inserting a sequence from the first hydrophobic segment of the β_2 receptor into $\alpha_2 F \rightarrow N$. These results are consistent with a structural model in which Asn^{312} of the β_2 receptor and Phe^{412} of the α_2 receptor form important structural interactions with the first hydrophobic segment.

The arrangement of transmembrane segments predicted by these experiments is similar to that found in bacteriorhodopsin. This approach should

facilitate the formulation and testing of more-accurate models for this class of membrane proteins. (This work was funded by a grant from the National Institutes of Health.)

Receptor Biosynthesis

Dr. Kobilka's group has used a cell-free expression system to study the process by which receptors are translated, translocated into the endoplasmic reticulum membrane, and folded into a functional protein. The β_2 receptor is a type IIIb membrane protein having multiple membrane-spanning domains with an extracellular amino terminus, but lacking a cleavable signal sequence. Dr. Xiaoming Guan has determined that the amino terminus and first hydrophobic segment of the β_2 receptor are inefficiently translocated, resulting in a nonfunctional receptor. The addition of a cleavable signal sequence to the amino terminus of the receptor greatly enhances translocation of the amino terminus and results in the production of more functional receptor protein. The increase in expression of functional protein afforded by the signal sequence would not be expected to have physiologic significance, yet it remains to be explained why this class of membrane proteins has evolved with a less-efficient mechanism for translocation. Following translocation of the first hydrophobic domain, subsequent pairs of hydrophobic domains are inserted efficiently, and their insertion is not dependent on translocation of preceding domains.

Post-transcriptional Regulation of the β_2-Adrenergic Receptor

The transcripts for the β_2 receptor from all species studied thus far contain a small open reading frame (SORF) 5' to the coding sequence of the receptor. The peptide encoded by the SORF is well conserved across species. Dr. Tony Parola has obtained evidence that even though the SORF has a poor Kozak consensus sequence, it is efficiently translated in both a cell-free expression system and cultured cells. Translation of the peptide is linked to reduced synthesis of β_2-receptor protein, because mutating the codon for the initiator methionine of the SORF leads to increased β_2-receptor expression.

Dr. Parola has observed that a synthetic peptide representing the product of the SORF is a potent inhibitor of translation in an *in vitro* expression system, suggesting that the product of the SORF negatively regulates β_2-receptor synthesis. However, the

reduced expression of the β_2 receptor may also be due to inefficiency in re-initiation of translation at the initiator methionine for the β_2 receptor following synthesis of the peptide encoded by the SORF. These experiments demonstrate that the β_2-receptor mRNA is bi-cistronic; the physiologic importance of the SORF in regulating β_2-receptor expression is yet to be determined.

Intracellular Trafficking of Adrenergic Receptors

Adrenergic receptors are plasma membrane proteins that permit extracellular catecholamines to influence cellular function. However, there is evidence that in some differentiated cells, such as neurons, adrenergic receptors may be targeted to specific domains on the plasma membrane. Furthermore, some adrenergic receptors are reversibly internalized following agonist stimulation. Little is known about the mechanism by which receptors are targeted to specific domains or how targeting is modulated by agonists.

Dr. Mark von Zastrow has been using immunocytochemical techniques to study adrenergic receptor targeting and intracellular trafficking. Following agonist stimulation, the β_2-adrenergic receptor undergoes rapid (within minutes) reversible internalization into an endosomal compartment that also contains the transferrin receptor. In contrast, the α_2 receptor undergoes a much slower internalization (hours), which is much less extensive. When both receptors are expressed in the same cell they are predominantly found in the plasma membrane. Following stimulation by a common agonist (epinephrine or norepinephrine), up to 75% of β_2 receptors are internalized within 15 minutes, while no significant redistribution of α_2 receptors is noted.

In addition to these differences in intracellular trafficking, Dr. von Zastrow has observed differences in targeting of two highly homologous subtypes of the α_2 receptor. The mouse homologue of the human α_2 receptor localized on chromosome 10 is found predominantly on the plasma membrane, while that localized on chromosome 4 is found predominantly in intracellular vesicles. The biological significance of these differences in targeting and trafficking is not known; however, these results suggest that in addition to structural determinants for ligand binding and G protein activation, receptors can be distinguished by determinants that differentially link receptors to cytoskeletal and membrane transport proteins. Future research will attempt to determine physiologic significance of differential sorting of receptors and to identify proteins involved in regulating the trafficking and agonist-mediated internalization of receptors.

Dr. Kobilka is also Assistant Professor of Cardiology and of Molecular and Cellular Physiology at Stanford University School of Medicine.

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REPLICATION AND PATHOGENESIS OF RNA VIRUSES

Michael M.-C. Lai, M.D., Ph.D., *Investigator*

RNA viruses, which include many common human and animal pathogens, provide important insights into general and unique features of the molecular biology of normal cells. Dr. Lai's laboratory has been studying two different RNA viruses that cover two extremes of the whole spectrum of RNA viruses.

Molecular Biology and Pathogenesis of Hepatitis Delta Virus

Hepatitis delta virus (HDV) is associated with a severe form of hepatitis with a high probability of developing chronic hepatitis and liver cirrhosis. HDV contains the smallest viral genome among animal viruses and thus must utilize its genetic information economically and rely on the host cells for its own replication. The genome is a single-stranded, circular RNA of 1.7 kb that may have been evolved from viroids, a plant pathogen. Dr. Lai and his colleagues demonstrated that the HDV RNA contains a "ribozyme" activity that can autocatalytically cleave and ligate itself. They have now characterized the *in vitro* and *in vivo* sequence requirement of this catalytic activity, which represents a new class of ribozyme. Furthermore, they have demonstrated that a cell-specific factor may modulate the ribozyme activity, and that the ribozyme is required for HDV RNA replication, which is carried out by cellular RNA polymerase II via a double rolling circle model.

HDV RNA encodes a single protein, hepatitis delta antigen (HDAg), from its antigenomic strand. This protein is the signature protein of HDV and is required for HDV RNA replication. Dr. Lai's laboratory has identified it to be a nuclear phosphoprotein with an RNA-binding activity specifically binding HDV RNA. The structure of HDAg has been dissected, and several functional domains have been identified. The first is a nuclear localization signal, which consists of two discontiguous basic amino acid–rich sequences. The second is an RNA-binding signal, which is composed of two discontiguous arginine-rich motifs (ARMs). The third is a leucine zipper–like sequence that promotes the oligomerization of HDAg. Dr. Lai and his colleagues have demonstrated that all of these activities are required for the replication of HDV RNA. They have proposed a model for the mechanism of action of HDAg, developed monoclonal antibodies specific for HDAg, and identified a potential domain important for the transcription function of HDAg. HDAg may be important for directing cellular enzymes to carry out RNA-dependent RNA synthesis.

Additional features of HDV biology were studied, including the heterogeneity and evolution of HDV RNA, which can be correlated with viral pathogenicity. HDV RNA has a high mutation rate, which may be responsible for the fluctuation in the clinical course of delta hepatitis.

Molecular Biology and Pathogenesis of Coronaviruses

Coronaviruses include a group of viruses that cause the common cold in humans and a variety of severe gastrointestinal and respiratory diseases in animals. One type also causes symptoms similar to those of multiple sclerosis, thus providing a model system for studying that disease. Dr. Lai's laboratory has demonstrated that mouse hepatitis virus (MHV), one of the prototype coronaviruses, contains an RNA genome that is 31,000 nucleotides long, which is the longest viral RNA known to exist in nature. This RNA contains various genes encoding viral structural and nonstructural proteins, whose properties and biological activities are currently being studied in Dr. Lai's laboratory.

The laboratory has demonstrated that MHV employs a unique discontinuous transcription mechanism that fuses a leader RNA to genes located some distance downstream to synthesize mRNAs. This transcription mechanism is an alternative to the conventional RNA-splicing mechanism seen in most mammalian cells. During the past year, Dr. Lai and his colleagues have continued to unravel the details of this mechanism. They have identified new transcription initiation sites and characterized the structure of template RNAs. This mechanism allows the leader sequence to control the expression of various viral genes. Thus, by altering the sequence of the leader RNA, the composition of viral proteins may vary. One most notable example is the hemagglutinin-esterase (HE) protein, whose presence is variable from virus to virus. Dr. Lai's laboratory has demonstrated that this protein is rapidly lost as virus evolves, and as a result the biological properties of the virus change. This rapid evolution of a viral protein is unique among viruses.

Dr. Lai's laboratory previously discovered the phenomenon of RNA recombination in coronavirus. Although once considered to be rare, genetic recombination has in recent years been increasingly

recognized as an important biological phenomenon and evolutionary tool for RNA viruses. During the past year the laboratory developed an RNA recombination system in which recombination occurs between viral genomic RNA and a transfected RNA fragment. This system not only provides insight into the mechanism of RNA recombination but may also prove to be a new genetic engineering tool for RNA viruses.

Dr. Lai and his colleagues are also interested in the neuropathogenesis of MHV, which is a model system for neurotropic viral infection. They examined the question of virus receptor and virus entry mechanisms in the central nervous system of mice and noted that MHV utilizes two different carcino-embryonic antigens (CEAs) as alternative receptors in liver or brain—the first virus to be shown to utilize two different receptors in different tissues. A resistant mouse strain was discovered that has a functional viral receptor but is resistant to viral infection. Furthermore, they found several cell lines that have functional viral receptors but are selectively resistant to certain MHV strains. Thus they have identified the requirement of a second cellular factor for virus entry into cells. This factor may interact with the viral receptor and control the target cell specificity of the virus. The nature of this second factor is being studied in Dr. Lai's laboratory. (This part of the work is supported by a research grant from the National Institutes of Health.)

Dr. Lai is also Professor of Microbiology and Neurology at the University of Southern California School of Medicine, Los Angeles.

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STRUCTURE AND REPLICATION OF INFLUENZA VIRUS AND PARAMYXOVIRUSES

ROBERT A. LAMB, PH.D., SC.D., *Investigator*

A major focus of Dr. Lamb's laboratory is to understand the structure and function of the integral membrane proteins encoded by influenza virus and the paramyxovirus SV5 (simian virus 5). In addition, the laboratory is investigating the mechanism by which these seven integral membrane proteins are transported in the exocytotic pathway to the cell surface. They provide a diverse group of differing prototype membrane proteins that span the membrane once and are known as type I, type II, and type III integral membrane proteins.

Membrane Protein Orientation Signals

Dr. Lamb and his colleagues are examining the signals necessary to establish the proper orientation of integral membrane proteins in a lipid bilayer. The paramyxovirus hemagglutinin-neuraminidase (HN) polypeptide, a model type II membrane protein, contains an internal uncleaved signal/anchor (S/A) and is oriented in the membrane with an amino-terminal cytoplasmic domain and carboxyl-terminal ectodomain. One of the major factors in establishing the orientation of this protein is the presence of positively charged residues flanking the hydrophobic membrane-spanning domain to retain this region of the protein in the cell cytoplasm. To test further the roles of amino-terminal positively charged residues in directing the HN membrane topology, the three arginine (Arg) residues within the 17–amino acid amino-terminal domain were systematically converted to a glutamine or glutamate, and the topology of the mutant proteins was examined after expression in CV-1 cells. The data indicate that 1) each of the amino-terminal Arg residues contributes to the signal directing proper HN topology, since substitutions in any of the three positions resulted in ~13–23% inversion into the N_{exo} form; 2) substitutions in the Arg directly flanking the S/A domain resulted in slightly more inversion than those that were located more distally; and 3) substitution with a negatively charged glutamate led to more inversion than did replacement with an uncharged glutamine.

Virus-mediated Cell-to-Cell Fusion

Although it has been well established that the paramyxovirus SV5 F protein when expressed in tissue culture cells can mediate syncytium formation, much evidence reported in the literature indicates that for other paramyxoviruses to cause fusion, coexpression of the attachment protein (HN) is also required. Dr. Lamb and his colleagues have investi-gated this further by expressing the SV5 F and HN proteins individually or by coexpression in CV-1 cells with SV40-based vectors and recombinant vaccinia viruses. The extent of detectable fusion in a syncytium formation assay was found to be affected by the expression system used. In addition, when HN was coexpressed with F, the expression vector system influenced the contribution of HN in forming syncytia. When the abilities of the SV5, human parainfluenza virus type 3, and Newcastle disease virus F proteins to cause fusion (when expressed alone or coexpressed with HN) were directly compared using the SV40-based vector system in CV-1 cells, the F proteins exhibited various degrees of fusion activity independent of HN expression, but the formation of syncytia could be enhanced to a different extent by the coexpression of the homotypic HN protein.

Internalization and Degradation of Glycoproteins

The SV5 HN glycoprotein expressed at the cell surface is internalized and degraded in lysosomes. HN lacks the typical aromatic amino acid residue in its cytoplasmic tail that has been found to be necessary for the internalization of several well-characterized receptor molecules. To investigate the nature of the signal encoded within the HN molecule that specifies internalization and degradation, chimeric molecules have been constructed between HN and another type II integral membrane protein that is not internalized, influenza virus neuraminidase. The data indicate that the HN transmembrane domain specifies internalization and targeting to lysosomes. Dr. Lamb and his colleagues are attempting to analyze the rate of internalization of HN and to determine whether it follows the clathrin-coated vesicle pathway. The project described above was supported by a grant from the National Institute of Allergy and Infectious Diseases, National Institutes of Health.

Influenza Virus M₂ Protein Has Ion Channel Activity

The influenza virus M_2 is a small (97–amino acid) integral membrane protein that is minimally a disulfide-linked tetramer. Circumstantial evidence based on the sensitivity of influenza virus to the drug amantadine hydrochloride, the coupling of antiviral action to the M_2 transmembrane domain, and the premature acid-induced conformational change in

the viral hemagglutinin in the presence of the drug led to the suggestion that M_2 is an ion channel that is required in virus particles to facilitate the viral uncoating process in secondary endosomes. To examine directly for ion channel activity, Dr. Lamb and his colleagues collaborated with Dr. Lawrence Pinto (Northwestern University) and expressed wild-type M_2 protein in oocytes of *Xenopus laevis*. The M_2 protein was shown to have an associated ion channel activity selective for monovalent ions, and the antiviral drug amantadine hydrochloride significantly attenuated the inward current induced by hyperpolarization of oocyte membranes. Mutations in the M_2 membrane-spanning domain that confer viral resistance to amantadine produced currents that were resistant to the drug. The M_2 protein bears no similarity to the structure of most ion channels cloned to date. Thus to eliminate the possibility that M_2 is a regulatory protein that activates a normally silent channel endogenous to oocytes, the currents of altered M_2 proteins were measured. That these currents showed a particular pattern for each mutant suggests that the channel pore is formed by the transmembrane domain of the M_2 protein, and thus it is a channel *per se*. Significantly, for the presumed role of the virion ion channel in virus uncoating in the acidic environment of secondary endosomes, the wild-type M_2 channel was found to be regulated by pH. The laboratory is currently performing a detailed structure-function analysis of this unusual ion channel. It is also testing whether other small integral membrane proteins encoded by viruses have a similar biological activity. Discrete aspects of this project are supported by a grant from the National Institute of Allergy and Infectious Diseases, National Institutes of Health.

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ADRENERGIC RECEPTORS

Robert J. Lefkowitz, M.D., *Investigator*

The major themes of research in this laboratory are the elucidation of the molecular properties and regulatory influences that characterize the receptor binding sites for catecholamines such as epinephrine and norepinephrine. These receptors can be categorized into two broad classes, α- and β-adrenergic receptors, that mediate the effects of catecholamines and related drugs on a wide variety of physiological processes. These adrenergic receptors are, in turn, prototypic of a much broader group of receptors coupled to guanine nucleotide regulatory proteins that mediate the effects of many agents on a remarkably diverse array of physiological functions.

The function of G protein–coupled receptors, such as the β-adrenergic receptor, is rapidly regulated by phosphorylation and dephosphorylation reactions involving several distinct protein kinases. In addition to the cAMP-dependent protein kinase, this unique system involves a family of G protein–coupled receptor kinases and the arrestin proteins.

Thus far three kinases and three arrestin proteins have been defined by biochemical, functional, and molecular cloning studies. Dr. Lefkowitz and his colleagues have cloned and sequenced full-length cDNAs for rhodopsin kinase, the enzyme that regulates rhodopsin function in the visual cycle, as well as the two distinct isoforms of βARK (β-adrenergic receptor kinase), βARK1 and βARK2, that regulate the function of the β-adrenergic receptor and other G protein–coupled receptors. Only the conformationally active forms of the various G protein–coupled receptors are substrates for the kinases. These enzymes phosphorylate the receptors on their carboxyl-terminal cytoplasmic tails. This leads to the binding of an arrestin protein to the receptor that thereby sterically interdicts signal transduction to the G protein, providing a means for rapid quenching of the stimulus.

In both the rhodopsin kinase and βARK systems, the enzymes are predominantly cytosolic but rapidly translocate to the membrane upon stimulation of the receptor. Until recently the molecular basis for this stimulus-orchestrated compartmentation of the enzymes was unknown. Inspection of the sequences of the cloned enzymes reveals that rhodopsin kinase, but neither of the βARKs, possesses a CAAX box at its carboxyl terminus (cysteine/aliphatic/aliphatic/any amino acid). This carboxyl-terminal signature sequence specifies a series of three interrelated post-translational modifications. These include isoprenylation of the cysteine by a C-15 farnesyl or C-20 geranylgeranyl moiety, followed by proteolysis of the last three amino acids and then carboxyl methylation of the now-terminal cysteine residue.

To test the role of rhodopsin kinase isoprenylation in its translocation, Dr. Lefkowitz and his colleagues prepared and expressed three different cDNAs, including the wild-type rhodopsin kinase, which ends with the residues CVLS, specifying farnesylation; another rhodopsin kinase ending in CVLL, which specifies geranylgeranylation; and a third in which the cysteine was mutated to serine, which is not isoprenylated at all. When the cDNAs were expressed in COS-7 cells, it could be documented that they specified the production of enzymes that were isoprenylated or not in the expected fashion. Moreover, whereas the C15 and C20 forms of the enzyme showed approximately equal biological activity to phosphorylate rhodopsin, the nonprenylated form of the enzyme was markedly impaired in this activity (<30% activity). Moreover, Dr. Lefkowitz and his colleagues were also able to measure the photon-induced translocation of the

enzyme in an assay developed for this purpose utilizing rhodopsin-containing rod outer segment membranes. Only the farnesylated form of rhodopsin kinase attached to the membrane-bound rhodopsin when stimulation with light was provided. Thus the geranylgeranylated form was largely membrane bound to begin with, and the nonprenylated form, which was not membrane bound in the absence of light, also failed to translocate even upon illumination. These data suggest that the isoprenylation of rhodopsin kinase plays a crucial role in mediating its light-induced translocation to rhodopsin-containing membranes and in supporting its biological activity.

The βARK does not contain a CAAX box and hence is not prenylated. However, Dr. Lefkowitz and his colleagues observed that if they prepared chimeric forms of βARK containing CAAX boxes, which therefore could be prenylated, their enzymatic activity increased by about threefold, similar to the results obtained with rhodopsin kinase. Moreover, the isoprenylated forms of the enzyme were better able to associate with receptor-containing vesicles. These data suggested that perhaps isoprenylation might in some way be involved in the translocation of βARK as well.

The γ subunit of the heterotrimeric G proteins, with which the receptors interact, are geranylgeranylated and are thought to play a role in anchoring the $\beta\gamma$ complex of the G proteins in the cell membrane. The laboratory showed that bovine brain $\beta\gamma$ subunits have a very marked stimulatory effect on the enzymatic activity of βARK1 and βARK2 (>10-fold), which is apparent when their ability to phosphorylate membrane receptors is examined but not when their ability to phosphorylate simple peptides is measured. Moreover, the $\beta\gamma$ subunit physically interacts with the βARK enzyme and mediates its attachment to receptor-containing membranes. The sites of interaction on the βARK enzyme are located in the last 200 amino acids, as documented by physical interaction studies between $\beta\gamma$ and GST fusion proteins containing the relevant sequences of βARK.

These new results indicate an important role for isoprenylation of proteins in mediating the stimulus-driven translocation of G protein–coupled receptor kinases. For rhodopsin, the isoprenyl group is found on the rhodopsin kinase, whereas for βARK it is donated by the $\beta\gamma$ subunit of G proteins. These $\beta\gamma$ subunits become available only when they are dissociated from α_s by interaction of G protein with receptor. Thus even as the G protein is being activated to mediate receptor signaling, it is providing the crucial $\beta\gamma$ subunit that helps to target βARK to

its receptors and desensitize the system. This provides yet another example of the elegant and interrelated feedback regulatory pathways that regulate transmembrane signaling events mediated by G protein–coupled receptors.

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PATTERN FORMATION AND GERM CELL DETERMINATION IN THE *DROSOPHILA* EMBRYO

RUTH LEHMANN, PH.D., *Assistant Investigator*

Essential cues for the establishment of the embryo's body pattern are provided to the egg cell during its maturation in the mother. Classical experiments that involved direct manipulation of early embryos indicated that some maternal information is localized within the egg cell. To understand how gene products can be spatially restricted within a single cell, Dr. Lehmann and her colleagues have focused on a specialized cytoplasmic region that forms during oogenesis at the posterior pole of the *Drosophila* oocyte. Nuclei that migrate into this cytoplasmic region during early embryogenesis become determined to the germ cell fate.

In addition to its role in germ cell determination, the posterior pole plasm also harbors a morphogenetic signal required at a distance for the normal development of the embryonic abdomen. Removal of this signal either by experimental manipulation or by mutation results in the lack of abdominal segments in the embryo. At least 10 maternal-effect genes (*nanos, pumilio, oskar, staufen, cappuccino, spire, mago nashi, vasa, valois,* and *tudor*) are involved either in the assembly of the pole plasm or in the synthesis, activity, and localization of the abdomen-specifying signal. All 10 maternal-effect genes share the abdominal phenotype, and all genes except *nanos* and *pumilio* affect germ cell formation.

Abdomen Formation and Mirror Images

The abdomen-specifying signal, encoded by the *nanos* gene, is localized in the posterior pole plasm. Charlotte Wang and Laura Dickinson showed that *nanos* RNA is synthesized during oogenesis and is localized to the posterior pole of the freshly laid egg. It is not localized to the posterior pole in those eight mutants that lack pole plasm. The abdominal segmentation defect of these mutants can thus be attributed to a lack of localized *nanos* activity.

The Nanos protein is distributed in a posterior-to-anterior concentration gradient. This protein also inhibits the activity of another maternally derived protein, encoded by the *hunchback* gene. The Hunchback protein is a repressor molecule that blocks transcription of genes within the embryo. By establishing a concentration gradient of Hunchback protein complementary to that of Nanos, *nanos* indirectly promotes activation of the first tier of embryonic genes required for abdomen formation.

To test whether a localized source of the Nanos protein is sufficient to change polarity and pattern of the embryo, Dr. Elisabeth Gavis used a mix-and-match strategy to express this protein at a new position in the embryo. First, Dr. Gavis showed that the 3'-untranslated region (3'-UTR) of *nanos* RNA contains an RNA localization signal and that this signal is sufficient to trigger localization of a reporter RNA to the posterior pole of the embryo. Then she removed the 3'-UTR of *nanos* and replaced it with the 3'-UTR from the *bicoid* gene, which is responsible for pattern formation in the head region of the *Drosophila* embryo. (Previously Dr. Paul Macdonald and Dr. Gary Struhl [HHMI, Columbia University] had shown that the 3'-UTR of *bicoid* RNA contains sequences necessary for localization of *bicoid* RNA to the anterior pole.) Finally, Dr. Gavis transferred the altered gene into flies and observed the effects on embryogenesis.

All females carrying the altered gene produce headless embryos with two abdomens in mirror

image. Thus, by swapping the 3'-UTR, *nanos* mRNA is mislocalized to the anterior pole and the Nanos protein gradient is set up at both ends of the embryo (because the mutant flies also carry the normal *nanos* gene), resulting in two complete abdomens. This experiment proved that a local source of the Nanos protein can establish polarity in the embryo. (This project is supported by a grant from the National Institutes of Health.)

Germline Determination

The other major interest of Dr. Lehmann and her associates is the origin of germ cells. The researchers want to know how germline tissues become different from other tissues. All animal species have some mechanism for setting aside germ cell precursors early in embryogenesis. Somehow these cells are protected from the signals that induce other cells to enter specific developmental pathways; thus they retain the ability to contribute to the generation of an entirely new organism.

At least eight genes (*oskar, staufen, cappuccino, spire, mago nashi, vasa, valois*, and *tudor*) are required for the localization of both the abdominal signal encoded by the *nanos* gene and a yet-unidentified signal that specifies the germ cell fate at the posterior pole. Molecular analysis indicates that the products of these genes act in a stepwise fashion to promote posterior pole plasm assembly. The Lehmann group and others have worked out the order of these steps; for example, the *staufen* gene product is required for localization of the *oskar* gene product, and both in turn are required for localization of the *vasa* gene product to the posterior pole. This pathway can be summarized as follows:

cappuccino,
spire → *staufen* → *oskar* →

vasa → *tudor* → *nanos* → abdomen
? → germ cells
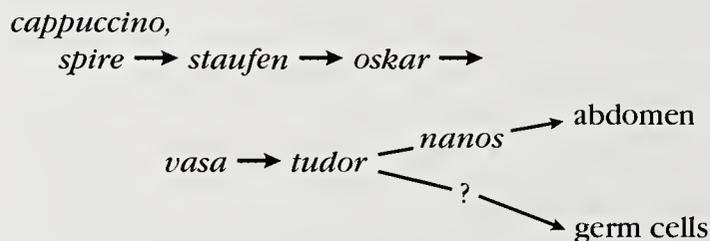
The same mix-and-match strategy described for *nanos* has allowed Dr. Anne Ephrussi to test this stepwise assembly model. Here the target gene was *oskar*, which acts in the middle of the pole plasm pathway. Dr. Ephrussi identified the *oskar* RNA localization signal within the 3'-UTR of *oskar* and replaced it with that of *bicoid*. Insertion of this chimeric gene into flies results in females that produce embryos with functional germ cells at both ends. Mislocalization of Oskar also leads to mislocalization of the abdominal signal *nanos* to the anterior. As a consequence these embryos form two opposing gradients of Nanos protein and develop two abdomens in mirror image.

A genetic analysis further shows that ectopic germ cell formation and anterior localization of *nanos* RNA require the activity of *oskar, vasa*, and *tudor*, while other genes, like *staufen, cappuccino, spire, valois*, and *mago nashi*, are not required. Thus these latter genes fulfill accessory functions for localizing the products of genes such as *oskar* to the posterior pole or for maintaining a functional germ plasm at this pole. These experiments have narrowed the number of genes directly involved in germ cell determination. Nevertheless the primary signal for germ cell determination still awaits identification. (This project is supported by the National Institutes of Health and the Packard Foundation.)

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MOLECULAR MECHANISMS OF EMBRYONIC INDUCTION

RICHARD L. MAAS, M.D., PH.D., *Assistant Investigator*

A central theme in vertebrate organogenesis is an interaction between two apposed cell layers, resulting in the morphologic transformation of one or both cell types. Such inductive interactions are involved in the development of many vertebrate organs. Dr. Maas's laboratory has focused on the role that transcription factors of the homeobox and paired box classes play in controlling inductive processes in two developmental systems, the formation of the vertebrate eye and the kidney. Both organs are formed on the basis of inductive interactions, and the same classes of gene products are implicated in the formation of both organs. A long-term goal is to dissect the genetic pathways involved in the formation of these two organs by identifying genes whose expression is regulated by *Hox* or *Pax* genes. An ancillary goal is to gain insight into pathogenesis of human birth defects involving the eye and the kidney.

Role of *PAX6* in Ocular Development

The vertebrate eye forms from an initial outgrowth of the forebrain, the diencephalon, which forms the optic vesicle and subsequently the optic cup. The optic cup comes to lie in apposition to the surface ectoderm, causing it to thicken and invaginate, forming the lens vesicle and then the lens. In the mouse, a semidominant mutation called *Small eye* exists in which this basic process is disturbed: heterozygotes have microphthalmia, small lenses, and absent or missing anterior chambers; homozygotes have completely absent eyes and noses. The ocular phenotype is consistent with a primary defect in lens induction, since transgenic mice whose lenses are ablated during embryogenesis have a phenotype strikingly similar to the *Small eye* mutation.

Dr. Maas's laboratory is studying the *Small eye* mutation as a way to study the role of paired box and homeobox transcription factors in inductive processes. The murine homeobox- and paired box–containing gene, *Pax-6*, has been mapped to a region near the *Small eye* locus on mouse chromosome 2. To test the hypothesis that *Pax-6* might be involved in the *Small eye* phenotype, the laboratory cloned the human *PAX6* gene in order to test its involvement in a disorder of human ocular development, aniridia, which has been proposed as homologous to the *Small eye* mutation. One rationale for choosing the human gene for study was that the number of *Small eye* alleles is limited to five, two of which involve large deletions. In contrast,

aniridia occurs at a frequency of ~1/64,000, and new mutations account for about one-third of aniridia cases. Thus there are a large number of different aniridia alleles, which could prove powerful in dissecting parts of the protein important for function.

To explore the role of *PAX6* in ocular development, Dr. Maas and his co-workers cloned the human *PAX6* gene and localized it to 11p13, the map location of aniridia. Work from the laboratory of Dr. Grady Saunders (University of Texas, Houston) has provided evidence that *PAX6* is involved in aniridia, by localizing *PAX6* to a smallest region of overlap of 70 kb. The human *PAX6* cDNA encodes a 422–amino acid protein with both homeobox and paired box domains, as well as a serine- and threonine-rich carboxyl terminus that could function as a transcriptional activator. This extraordinarily conserved gene is 100% identical at all 422 amino acids to the mouse protein and 96% identical to the homologous zebrafish gene at the amino acid level. Results from other laboratories have shown that *Pax-6* is expressed in the optic vesicle, lens, olfactory bulb, and hindbrain.

Dr. Maas's group determined the complete genomic structure of the human *PAX6* gene. This gene, which spans ~30 kb of genomic DNA, has 14 different exons and is the most complex *Hox* or *Pax* gene structure determined to date. One exon is alternatively spliced and, when utilized, inserts 14 amino acids into the paired domain, potentially changing the DNA-binding specificity of the protein. The sequence of all exon-intron boundaries has been determined, as well as that of the surrounding flanking sequences. This information has permitted the design of polymerase chain reaction (PCR) primers that amplify each individual exon of the human *PAX6* gene, making it possible to analyze it efficiently for mutations by the technique of single-stranded conformation polymorphism (SSCP) analysis.

Using genomic DNAs from 10 aniridia patients, members of the laboratory identified four independent mutations in the human *PAX6* gene in aniridia. These mutations include a 1-bp deletion in a splice donor site, a 7-bp insertion into the *PAX6*-coding region resulting in a frameshift, and a nonsense mutation in the homeodomain just prior to the third helix, which lies in the major groove of DNA and is thought to be primarily responsible for sequence recognition. This last mutation would result, at a minimum, in a protein that would be unable to bind

to DNA by its homeodomain. Although it is possible that these mutations could permit a partially functional protein to be made, it is more likely that they result in a complete loss of function. These results prove that mutations in *PAX6* account for aniridia, and that aniridia is the human counterpart to the *Small eye* mutation in mice since independently, it has been shown that the mouse *Pax-6* gene is mutated in *Small eye* mice. Surprisingly, Dr. Maas and his colleagues have not detected mutations in six other patients; experiments are under way to determine if these patients have mutations in the *PAX6* gene that were undetected by SSCP. Experiments are also under way to determine whether the *PAX6* gene product functions in inductive processes in the developing eye and, if so, how it functions.

Role of *Hox* Genes in Nephrogenesis

Previous experiments indicate that the ureteric bud must form properly in order for nephrogenesis to occur, since mutants such as the *limb deformity* mouse, in which its outgrowth is retarded, manifest a renal agenesis phenotype. Dr. Maas's laboratory has characterized a novel *Hox* gene that is expressed in the developing mouse kidney and has been provisionally identified as *Hox-1.8*. Analysis of cDNA clones for this gene has revealed an unexpectedly complex number of different transcripts that are related to each other by alternative splicing. This gene is expressed in the periureteral mesenchyme during mouse kidney development. Studies are under way to determine the function of *Hox-1.8* in nephrogenesis.

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CELL CYCLE–REGULATED PROTEIN KINASES

James L. Maller, Ph.D., *Investigator*

Two events mark the reproductive life of a cell: replication of genomic DNA in S phase and distribution of that replicated DNA to daughter cells at mitosis or M phase. Dr. Maller is interested in the molecular mechanisms involved in the decision to initiate mitosis, as well as the control of discrete events in M phase itself. The initiation of mitosis is signaled by activation of the protein kinase activity of the *cdc2* gene product. Prior to this activation the cell must pass a checkpoint in G_2 phase whose main function is to ensure that cdc2 kinase is not activated until DNA replication is complete or damaged DNA is repaired. In the past year substantial progress has been made in unraveling the biochemistry of this G_2 checkpoint. In addition, new insight into the mechanism of metaphase arrest at meiosis II has been gained.

Regulation of the *Xenopus* cdc25 Phosphatase

Previous work in this and other laboratories has shown that activation of cdc2 kinase occurs via dephosphorylation of tyrosine 15, an event mediated in *Schizosaccharomyces pombe* by the cdc25 phosphatase. Overexpression of cdc25 in *S. pombe* leads to entry into mitosis, even with incompletely replicated DNA, implying that cdc25 is involved in G_2 checkpoint control. To study cdc25 in a higher eukaryote, Dr. Tetsuro Izumi in this laboratory cloned and sequenced the *Xenopus* cdc25 phosphatase and studied its regulation in egg extracts that carry out both DNA synthesis and mitosis *in vitro*. Initial experiments showed that the amount of cdc25 protein does not change during the cell cycle, but there is a marked retardation in electrophoretic mobility on gels in both meiotic and mitotic M phases. Subsequent work showed the shift is due to periodic phosphorylation and, moreover, the phosphorylated form is considerably more active in dephosphorylating cdc2 kinase and elevating its protein kinase activity. This important work identified biochemically two additional elements in the G_2 restriction point: the protein kinase and protein phosphatase that control the activity of the cdc25 phosphatase. *In vitro* studies showed that either protein phosphatase 1 (PP1) or 2A could dephosphorylate and deactivate cdc25. Analysis of PP1 activity during the cell cycle revealed periodic oscillations: activity is high in M phase and S phase but low just prior to cdc2 activation and during exit from mitosis. The existence of two peaks of PP1 activity during the cell cycle is consistent with previous genetic work in yeast, in which altered expression of PP1

had phenotypes in cell cycle phases that correspond to the peaks of PP1 activity.

These results indicate that the initiation of cdc25 activation is regulated at least in part by changes in PP1 activity. PP1 is also regulated by the presence of incompletely replicated DNA, since its activity remains high when the cell cycle is arrested in S phase by inhibitors of DNA synthesis. It is likely there are additional targets besides cdc25 for regulation by PP1. Elucidating the mechanism by which PP1 activity oscillates merits urgent attention, as does identification of the protein kinase(s) responsible for phosphorylating and activating cdc25.

The cdk2 Protein Kinase and Meiotic Metaphase Arrest

Genetic evidence in yeast indicates that the *cdc2* gene controls both the G_1 and G_2 restriction point. However, in higher eukaryotes, several genes closely related to cdc2 (termed cdks, for cyclin-dependent kinases) have been cloned by various laboratories, and a role for them in G_1/S regulation has been suggested. The best-studied cdk is cdk2 in *Xenopus*. The mRNA for this gene is stored in the oocyte and becomes translated only during the meiotic cell cycles of oocyte maturation, although the kinase activity of cdk2 persists into the early embryonic cell cycles. *Xenopus* cdk2 is active as a protein kinase only in a complex with other proteins, whose identity is unknown but is distinct from cyclins A and B.

This year substantial progress was made in understanding the role of cdk2 in the meiotic cell cycles of *Xenopus*. In meiosis II, cdk2 kinase activity rises, although the polypeptide accumulates earlier, in meiosis I. To determine if elevated cdk2 activity is important for meiosis, Dr. Brian Gabrielli designed antisense oligodeoxynucleotides against cdk2 and showed that injection of these into oocytes prior to stimulation of meiotic maturation abolished expression of cdk2. No effect of cdk2 ablation was observed on cdc2 kinase activity in meiosis I. In meiosis II, however, metaphase arrest failed to occur and the oocytes proceeded directly from meiosis II into the first embryonic cell cycle. Important control experiments showed that injection of purified cdk2 into oocytes along with the antisense oligonucleotides restored a metaphase II arrest with elevated cdc2 kinase activity. It had been known for some time that metaphase II arrest requires the expression of the c-mos proto-oncogene kinase plus one or more newly synthesized proteins. The present results suggest cdk2 as one of the new proteins required along with c-mos for meiosis II metaphase arrest. The cooperation between the c-*mos*^{xe} and cdk2 protein kinases in meiosis II arrest is another example of the complex network linking cell growth control by oncogenes with cell cycle control elements.

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CONTROL OF CELL GROWTH AND DIFFERENTIATION BY TRANSFORMING GROWTH FACTOR-β

Joan Massagué, Ph.D., *Investigator*

The proliferation of cells is controlled by a balance of positive and negative signals. The machinery that conveys growth inhibitory signals is similar in design to that which signals cell growth. Both involve factors that circulate between cells and membrane growth factor receptors that are coupled to signal transduction circuitry inside the cell. The signals carried by growth-promoting factors have been extensively studied for the past two decades. The growth inhibitors, however, have come to the attention of Dr. Massagué only recently. Yet they include some of the most widespread and versatile regulators of cell growth and differentiation. Some are implicated in processes of development and tissue repair, and their study may illustrate ways to constrain the unrestricted growth of cancer cells. In addition, given their multifunctional nature, they may serve to dissect the signaling networks that are coupled to regulation of cell cycle progression, differentiation, senescence, and death.

Multifunctional Growth Inhibitors and Their Receptors

Transforming growth factor-β (TGF-β) is a prototypic growth inhibitory polypeptide. It belongs to a large family of growth and differentiation factors that also includes the activins, the bone morphogenetic proteins (BMPs), the Müllerian inhibiting substance (MIS), and others. The evolutionary conservation of these factors is unusually strict, and they are broadly multifunctional. For example, TGF-β can inhibit cell proliferation, regulate cell differentiation, and influence supracellular organization by controlling cell adhesion, migration, and extracellular matrix formation.

Over the past year, research in Dr. Massagué's laboratory has centered on identifying and cloning receptors for TGF-β and related factors. Each of these factors binds to various classes of membrane proteins that can be visualized by receptor affinity-labeling assays. One class consists of transmembrane receptors with a protein-serine/threonine kinase domain in the cytoplasmic region. One member of this class binds TGF-β, whereas others bind activin. It is anticipated that additional members of this family yet to be cloned bind the other TGF-β–related factors, BMPs and MIS. Like the factors they bind, these receptors exist in many isoforms, each probably representing a discrete adaptation to achieve optimal control of cell functions.

In contrast to the large class of growth factor receptors with protein-tyrosine kinase activity, the serine/threonine kinase TGF-β receptor requires another TGF-β–binding component to signal. Dr. Massagué and his colleagues are examining the hypothesis that the serine/threonine kinase receptors signal as part of heteromeric receptor complexes.

Another recently cloned TGF-β receptor type is interesting for other reasons. This protein, betaglycan, is thought to act as a helper of the signaling receptors. Rather than mediating cell responses directly, betaglycan appears to act as a regulator of cell access to TGF-β by either presenting this factor to the signaling receptors or storing it for later use by the cell. The structure of betaglycan is unusual for a growth factor–binding protein: it is a membrane-anchored proteoglycan that binds TGF-β through its core protein. Work is under way to map the portion of this molecule that binds TGF-β and to test its ability as a modulator of TGF-β activity.

The cloning of these genes makes it possible to explore in detail their structural and functional properties and to identify additional components of the TGF-β signal transduction system. Furthermore it should now be possible to determine to what extent the genetic loss of TGF-β receptors might cause loss of constraint in cell proliferation and contribute to oncogenic transformation.

Coupled Control of Cell Proliferation and Differentiation by TGF-β

By lengthening G_1 phase in the cell cycle, TGF-β can restrict or even arrest cell proliferation. Normally this growth inhibitory effect is reversible and ends upon removal of TGF-β from the medium. Growth inhibition might become permanent, however, if slow cell cycle progression induced by TGF-β facilitated cell commitment to terminal differentiation. Often cells that are competent to differentiate remain undifferentiated while exposed to proliferative stimuli and undergo differentiation if mitogenic stimulation passively subsides. Mitogens retain myoblasts in a proliferative state that interferes with the ability of MyoD1 and related transcription factors to initiate differentiation. However, myogenic differentiation in developing tissues occurs in environments where mitogens presumably abound, and differentiation in these tissues might depend on factors such as TGF-β that can actively override the inhibitory effect of mitogens.

Dr. Massagué and his colleagues found that TGF-β can counteract the inhibitory effect of a growth-promoting environment on rat skeletal myoblast differentiation. Induction of myogenic differentiation by TGF-β occurs with acute down-regulation of c-myc and Id, two transcription factors that promote cell cycle progression and antagonize myogenic differentiation. These findings suggest that TGF-β may act as a physiological inducer of myogenic differentiation. Its growth inhibitory effect may trigger terminal differentiation with permanent withdrawal from the cell cycle. Furthermore these results illustrate how the same factor can simultaneously affect cell proliferation and differentiation by controlling elements that operate in the crossroad of these two important processes.

Dr. Massagué is also Member of the Cell Biology and Genetics Program at Memorial Sloan-Kettering Cancer Center and Professor of Cell Biology at Cornell University Medical College Graduate School, New York City.

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TRANSCRIPTIONAL REGULATORY PROTEINS

Steven L. McKnight, Ph.D., *Investigator*

During the past year Dr. McKnight's laboratory has focused on the terminal differentiation of adipose cells. More than a decade ago Dr. Howard Green isolated a line of cultured mouse cells that could be induced to differentiate from a proliferative, fibroblastic phenotype into fat-laden adipocytes. The differentiation process undertaken by

these 3T3-L1 cells was determined by empirical methods to be regulated by three hormonal inducers: insulin, dexamethasone, and methylisobutylxanthine (a phosphodiesterase inhibitor). When exposed to this hormonal cocktail, otherwise proliferative 3T3-L1 cells initiate a differentiation program that culminates in the arrest of mitotic growth

and the elaboration of a specialized adipogenic phenotype.

It is well established that the process of terminal adipocyte differentiation entails ordered changes in gene expression. Dr. Daniel Lane and his colleagues (Johns Hopkins University School of Medicine) have identified genes induced during fat cell differentiation that encode a variety of enzymes and fatty acid–binding proteins required for the synthesis and storage of triglycerides, lipids, and fats. Surprisingly, these fat-specific genes are not the direct targets of the aforementioned hormonal inducers. The optimal differentiation program, for example, entails the initial exposure of 3T3-L1 cells to insulin, dexamethasone, and methylisobutylxanthine for a two-day period. Thereafter the last two hormones are removed from the culture medium, and only after an additional four days are the fat-specific genes induced. Apparently the inducing effects of dexamethasone and methylisobutylxanthine are somehow relayed during the process of terminal differentiation.

Several years ago Dr. McKnight and Dr. Ed Birkenmeier (a collaborative associate from the Jackson Laboratory) noted a correlation between adipocyte differentiation and the synthesis of a transcription factor called CCAAT/enhancer-binding protein (C/EBP). Neither the factor nor its encoding mRNA is present in proliferating 3T3-L1 preadipocytes. C/EBP is synthesized and accumulated to a substantial level, however, as the cells acquire their differentiated phenotype. Many of the fat-specific genes identified by Dr. Lane appear to be regulated by C/EBP. Binding sites for C/EBP occur in the promoters of many such genes, and evidence demonstrating a direct, activating role for C/EBP has been established in both test-tube and living cell assays.

Two experiments, one conducted by Dr. Lane's laboratory and the other by Dr. McKnight's laboratory, provided evidence confirming the role of C/EBP in fat cell differentiation. In the former case, antisense inhibition experiments were used to block the expression of C/EBP, and, hence, terminal adipocyte differentiation. Complementary experiments designed to express C/EBP prematurely during adipogenesis resulted in an accelerated differentiation program.

Although such studies firmly establish the essential role played by C/EBP in adipose conversion, they did not resolve the linkage between the hormonal inducers of differentiation and the activation of the adipogenic genetic program. Neither the synthesis nor activity of C/EBP is influenced by any of the three adipogenic hormones. An apparent breakthrough came, however, when Dr. Zhaodan Cao (HHMI Associate) discovered genes encoding two C/EBP-related proteins, C/EBPβ and C/EBPδ. Expression profiles of these genes correlate with the presentation and withdrawal of dexamethasone and methylisobutylxanthine. Moreover, it was shown that dexamethasone is a direct inducer of transcription of the gene encoding C/EBPδ and that methylisobutylxanthine is a direct inducer of the gene encoding C/EBPβ. Dr. McKnight and his colleagues have tentatively speculated that the β and δ isoforms of C/EBP represent relay switches that pass on the effects of their inducing, adipogenic hormones. Tests of this hypothesis are now under way. Should the notion turn out to be correct, the work will have provided a satisfying extension to the pioneering studies of Dr. Green, as well as the groundwork for future molecular biological studies on the problem of cell differentiation and specialization.

Dr. McKnight is also a staff member in the Department of Embryology at the Carnegie Institution of Washington, Baltimore, and Adjunct Professor in the Departments of Biology and of Molecular Biology and Genetics at the Johns Hopkins University School of Medicine. Effective in the next year, he will leave to assume the position of Research Director of Tularik, Inc.

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CELLULAR INTERACTIONS IN EMBRYOGENESIS

ROELAND NUSSE, PH.D., *Associate Investigator*

Research in the laboratory of Dr. Nusse is focused on the function of growth factors in early embryogenesis, in particular on a group of genes whose original discovery was related to their oncogenic properties. The prototype member of this group, *Wnt-1*, is an oncogene in mouse mammary cancer; normal expression of this gene is limited to early development. *Wnt-1* is part of a large family of related genes. All members of the mouse *Wnt* family encode secreted proteins closely related to the *Drosophila* segment polarity gene *wingless*. Dr. Nusse's group studies the function of this family of genes in both mammals and *Drosophila*.

Expression of Mouse *Wnt* Genes During Embryogenesis

Dr. Patricia Salinas, a fellow from the Leukemia Society, has further analyzed the expression of *Wnt-3* in the developing brain. In 9.5-day mouse embryos, *Wnt-3* is expressed in a restricted area of the diencephalon before any morphological signs of segmentation appear. Around embryonic day 11.5, *Wnt-3* expression becomes restricted to the D2 neuromere (dorsal thalamus). A mouse homologue of the *Drosophila Distal-less* gene is expressed in a nonoverlapping area immediately anterior to and abutting the *Wnt-3*–expressing domain, corresponding to the D1 compartment (ventral thalamus). In addition, *Wnt-3* is expressed in the midbrain-hindbrain region. In the adult mouse, *Wnt-3* is expressed in subsets of neural cells derived from the original areas of expression in the diencephalon. These results suggest that *Wnt-3* provides positional information for the formation of segmental boundaries and defines the regional identity of compartments within the central nervous system (CNS). The continued expression of *Wnt-3* in the adult mouse brain suggests a distinct role in the mature CNS.

In the brain of adult mice, *Wnt-3* expression is found in the dorsal thalamus. In addition, the gene is highly expressed in Purkinje cells of the cerebellum, which are located immediately adjacent to the granular layer. In the cerebellum from mice mutant at the *weaver* locus, where the migration of granular cells is disturbed, *Wnt-3* expression in the Purkinje cells decays prematurely.

The *wingless* Gene Family in *Drosophila*

The segment polarity gene *wingless* is thought to interact with a group of genes with a similar phenotype to control cell differentiation within individual segments of *Drosophila* embryos. Dr. Nusse's laboratory is particularly interested in the interaction of *wingless* with other segment polarity genes, one of which may encode the *wingless* receptor. At least one other segment polarity gene, *engrailed*, is under the control of *wingless* expression, and other segmentation genes are thought to mediate this control.

One interest of the laboratory is to establish functional assays for *wingless* by rescuing mutants with wild-type DNA constructs and to do structure-function analysis with site-directed mutants. Derek Lessing, a graduate student, has recently established the position of the 5′ end of the *wingless* transcriptional unit and has sequenced 2,500 base pairs of the *wingless* promoter. Marcel van den Heuvel and Cynthia Harryman have characterized the molecular nature of several existing *wingless* alleles. In all of the embryonic viable alleles analyzed, the encoded protein is unaltered. Embryonic lethal alleles, in contrast, have all undergone mutations in the protein-encoding domain of the gene, including deletions, point mutations of conserved residues, and P-element inserts. A temperature-sensitive mutation was shown to be a substitution of a conserved cysteine for a serine residue. Immunostaining of mutant embryos shows that the embryonic lethal alleles lead to either no *wingless* antigen or to an apparently nonsecreted form of the protein. Transfection of the mutant alleles into tissue culture cells followed by immunoprecipitation or immunostaining analysis supported these results. Wild-type protein is found on the cell surface or in the extracellular matrix, whereas mutant protein is retained in the endoplasmic reticulum (ER).

Dr. Lee Fradkin has generated epitope-tagged *wingless* variants to examine protein secretion in S2 cells and to purify the *wingless* protein from either eukaryotic or prokaryotic cells. In collaboration with Dr. Norbert Perrimon (HHMI, Harvard Medical School), Marcel van den Heuvel has examined the distribution of the *wingless* protein in embryos mutant for other segment polarity genes to identify gene products interacting with the *wingless* protein. The results suggest that the *porcupine* gene product plays a role in the secretion of the *wingless* protein. Further molecular analysis of these genes and their products is in progress.

To examine the consequences of ectopic *wingless* activity, Jasprien Noordermeer made transgenic flies that express *wingless* under the control of an *hsp70*

promoter (HS-*wg* flies). Ubiquitous *wingless* expression results in naked ventral cuticle, broadening of the *engrailed* domain in the posterior direction, and induction of endogenous *wingless* transcription. Jasprien Noordermeer and John Klingensmith have combined the HS-*wg* allele with loss-of-function mutations in various other segment polarity genes. The results of these double-mutant experiments point to essential functions for several genes in the *wingless* pathway. For example, it appears that the expression of *dishevelled* is essential to generate the naked cuticle and the ectopic expression of *engrailed*. In contrast, *armadillo* is not required for the broad *engrailed* expression induced by HS-*wg* but is necessary to maintain this pattern. John Klingensmith and Frank van Leeuwen are currently using specific antibodies and transfected cell lines to analyze the *dishevelled* gene product.

wingless is also part of a gene family in *Drosophila*. One of the *wingless*-related genes, D*Wnt-3*, is highly unusual. Its predicted protein is much longer than other *Wnt* proteins and contains long inserts at various sites. Dr. Fradkin has made an antibody specific for this member of the *Wnt* gene family. With *in situ* hybridization methods and with antibody stainings, it was found that D*Wnt-3* is expressed in the developing CNS of *Drosophila* embryos, with additional sites in the epidermis of gnathal segments. Experiments are under way to assess the role of these genes in embryogenesis by isolating mutants and by overexpression of the genes under the control of a heat-shock promoter.

Dr. Nusse is also Associate Professor of Developmental Biology at Stanford University School of Medicine.

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MECHANISM OF DNA REPLICATION

Michael E. O'Donnell, Ph.D., *Assistant Investigator*

Duplication of the genetic material is central to the life process of every cell. Although this need occur only once, it must be performed accurately to preserve the species. Dr. O'Donnell and his colleagues are studying the series of individual steps by which the genetic material is duplicated. The system being studied is the bacterium, *Escherichia coli*, which, like most organisms, stores its genetic information in the form of double-strand DNA. Duplication of the DNA, a process called replication, requires more than a dozen proteins. Ten of these are bound together into a multiprotein machine: one subunit of this machine is the actual DNA polymerase; another subunit is a 3′–5′ exonuclease that proofreads the product of the DNA polymerase. The multiprotein chromosomal replicase is called DNA polymerase III holoenzyme, referred to here as the "holoenzyme."

Both polymerase and exonuclease activities are found in most DNA polymerases, including the classic DNA polymerase I of *E. coli*, which mainly functions to repair damage to the DNA chromosome incurred during everyday life. It is the other eight subunits, or accessory proteins, of the holoenzyme that are unique to the chromosomal replicative polymerase. It seems likely that they each have an individual function in the process of duplicating a chromosome in which the two strands of double-stranded DNA are separated, and then each single strand is used as a template to make two new daughter duplexes. The process sounds simple, but is difficult; too little is known even to guess at individual functions for these eight accessory proteins. A combination of genetics and biochemistry is being used to identify the steps in the replicative process.

Genetics

Four genes that encode five subunits of the holoenzyme have been known for some time. The *dnaE* gene encodes α, the DNA polymerase; *dnaQ* encodes ϵ, the 3'–5' exonuclease; *dnaN* encodes the β subunit; and *dnaX* encodes both the τ and γ subunits (γ is produced from *dnaX* by a translational frameshifting mechanism). Each of these genes is essential for cell viability, as expected for subunits of the chromosomal replicase.

Dr. O'Donnell's laboratory has recently identified and sequenced the genes for the remaining five subunits: δ (*holA*), δ' (*holB*), χ (*holC*), ψ (*holD*), and θ (*holE*). The studies on *holB*, *holC*, *holD*, and *holE* were supported by a grant from the National Institutes of Health. These genes are now being used to determine the intracellular role of each subunit by constructing strains of *E. coli* that are missing one or more of these genes. Preliminary studies indicate that *holE* is not essential for cell viability. Work on the other genes is in progress. Strains that survive in the absence of a *hol* gene will be analyzed for growth defects. It seems possible that a subunit(s) of the holoenzyme could perform a specialized role such as proofreading one particular mismatch or interfacing with other cellular machineries for DNA repair, recombination, or mutagenesis. Therefore any nonessential subunit genes will be studied in a variety of genetic backgrounds to identify possible roles in fidelity, repair, recombination, or mutability.

Biochemistry

The holoenzyme is a remarkably rapid (750 nucleotides/s) and processive (>100,000 nucleotides/template-binding event) polymerase. This rapid and processive synthesis sets it apart from repair polymerases and distinguishes it as a chromosomal replicase. During the past two years the molecular mechanism of this remarkable speed and processivity of the holoenzyme has been elucidated and is described below.

The holoenzyme can be resolved into three components. One is a three-subunit subassembly called the core polymerase that contains the α (polymerase), ϵ (3'–5' exonuclease), and θ subunits. Another is the γ complex which has five subunits ($\gamma\delta\delta'\chi\psi$). The third component is the β subunit. The core polymerase is not a rapid and processive polymerase but instead is even slower and less processive than DNA polymerase I. The γ complex and β are both needed to confer the processive speed onto the core polymerase. They do so in the following fashion. The γ complex recognizes a primed template and then couples the energy of ATP hydrolysis to clamp a dimer of the β subunit onto the primed template. After this the γ complex may leave and the β dimer remains behind, bound to the primed template. This β dimer remains tightly bound to circular DNA, but upon linearizing the DNA, it slides freely off over either end. Hence the β dimer has mobility on DNA, and since it falls off linear DNA but not circular DNA it must bind the DNA by encircling it like a doughnut. The γ complex must function to assemble the β dimer around DNA. Recently, in collaboration with Dr. John Kuriyan (HHMI, Rockefeller University), the x-ray structure of the β dimer was solved. As expected, β is a ring-shaped protein with a central cavity of sufficient size to accommodate duplex DNA.

The β dimer, besides acting as a sliding clamp by encircling DNA, also binds directly to the core polymerase. Hence the fundamental basis for high processivity is a sliding clamp of β, which continuously holds the polymerase down to the primer template, thereby making it behave in a highly processive fashion. As the polymerase synthesizes DNA, it simply moves forward and pulls the β sliding clamp along with it. This project was supported by the National Institutes of Health, which is also supporting current investigations on how the γ complex couples ATP to the opening and closing of the β ring.

"Form follows function," and both structural and functional studies are important complementary avenues of investigation. Knowledge about the structure of the holoenzyme, the stoichiometry of its subunits, and the subunit-subunit contacts within it are important to understand how they perform their functions as a unit. To this end, all 10 subunits have been overproduced and purified. The α, ϵ, β, γ, and τ proteins have been overproduced by molecular cloning of their genes in the laboratories of Drs. Arthur Kornberg and Charles McHenry and Drs. Harrison Echols, Alvin Clark, and James Walker. Likewise, using the recently identified genes, Dr. O'Donnell's laboratory has overproduced and purified the δ, δ', χ, ψ, and θ subunits. Using these pure proteins, they have identified many subunit contacts and determined many stoichiometries, although the study is not yet complete. Among these contacts is one mediated by the τ subunit, which is a dimer in its native state. The τ dimer binds directly to the α subunit, the DNA polymerase. Since τ is dimeric, it binds two polymerase subunits. Hence, as predicted long ago by Dr. Kornberg, the holoenzyme has two DNA polymerase subunits, as expected for a replicase that must synthesize both strands of a duplex DNA chromosome.

The five-protein γ-complex particle is efficiently constituted from the five separate subunits. Functional studies show γ and δ are capable of assembling the β clamp, but they are inefficient in doing so and the δ' subunit is highly stimulatory, indicating an important role for δ' in assembling the β ring onto DNA. The 10-subunit polymerase holoenzyme is in the process of being assembled. These structural studies were supported by a grant from the National Institutes of Health.

Epstein-Barr Virus Replication

The Epstein-Barr virus (EBV) is the causative agent of infectious mononucleosis. After the initial infection, the EBV genome exists in a latent state as multiple copies of a 172-kb plasmid DNA in B lymphocytes. Latent replication of EBV is tightly regulated, occurring only once per division of the host cell and in synchrony with the host chromosomes. Dr. Bill Sugden's laboratory has identified the viral latent origin of replication (*oriP*) and the single EBV-encoded gene product (EBNA1; EBV nuclear antigen 1) essential for latent EBV replication. Dr. O'Donnell's laboratory has overproduced the EBNA1 protein in the baculovirus expression system.

Study of EBNA1 shows it induces a large DNA loop within the origin between the two essential elements of *oriP*, the family of repeat (FR) element and the dyad symmetry (DS) element. The FR element is thought to be an enhancer of replication initiation at the DS element. The loop induced by EBNA1 between these two elements is reminiscent of loops generated by transcriptional enhancer proteins, implying similar methods are used to activate replication. In another study, the EBNA1 protein was found to induce a structural distortion in the DS element, perhaps an initial step to set the stage for entry of the host replication proteins into the origin. These studies have been supported by the National Institutes

of Health. Study of the role of EBNA1 in regulated replication at *oriP* may further understanding of how eukaryotes regulate the replication of their chromosomes.

Dr. O'Donnell is also Associate Professor of Microbiology at Cornell University Medical College, New York City.

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REGULATION OF THE ADRENAL STEROIDOGENIC ENZYMES

KEITH L. PARKER, M.D., PH.D., *Assistant Investigator*

Dr. Parker's laboratory studies the mechanisms that regulate the adrenal steroidogenic enzymes. Steroid biosynthesis requires the concerted action of cytochrome *P*-450 enzymes that convert cholesterol to biologically active products. All steroidogenic cells express cholesterol side-chain cleavage en-

zyme (SCC), whereas steroid 21-hydroxylase (21-OHase) and 11β-hydroxylase (11β-OHase) are limited to adrenocortical cells. Treatment of adrenocortical cells with corticotropin (ACTH) coordinately increases the expression of all of these steroid hydroxylases; this induction is a major component

of the hormone's action to maintain steroidogenic competence. The objective of these studies is to define the factors that regulate the adrenal steroidogenic enzymes; in particular, the studies have focused on defining shared regulatory proteins that determine the coordinate expression of this gene network.

Mouse Homologue of the Nuclear Receptor *fushi tarazu* Factor I Regulates Steroidogenic Enzyme Gene Expression

Dr. Parker's laboratory has characterized the promoter regions of mouse genes encoding SCC, 21-OHase, and 11β-OHase. Initial studies showed that the 5'-flanking regions of these genes are sufficient for regulated expression and define key regulatory elements. Of particular interest, several promoter elements contain variations of an AGGTCA motif, suggesting they interact with the same protein. This protein, which was designated steroidogenic factor 1 (SF-1), interacts with promoter elements from all of the mouse steroidogenic enzymes and is selectively expressed in steroidogenic cell lines, establishing it as a likely determinant of coordinate, cell-selective expression of these essential genes.

The AGGTCA recognition sequence of SF-1 closely resembles that of several members of the nuclear receptor family, a superfamily of structurally related, regulatory proteins that mediate transcriptional induction by such diverse agents as steroid and thyroid hormones, vitamin D, and retinoic acid. On the assumption that the similar recognition motifs reflect structural similarities between nuclear receptors and SF-1, a Y1 adrenocortical cell cDNA library was screened with a probe comprising the DNA-binding domain of H-2RIIBP, one family member. One clone hybridized specifically to transcripts that are only expressed in steroidogenic tissues, the same pattern seen with SF-1. Moreover, expression of this cDNA as a glutathione *S*-transferase fusion protein yielded a protein that interacted with the steroidogenic regulatory elements in a manner indistinguishable from SF-1.

When the nucleotide sequence of the SF-1 cDNA was determined and compared with other sequences, a surprising finding emerged. This sequence is highly related to the nuclear receptor family member FTZ-F1, which regulates the *fushi tarazu* (*ftz*) homeobox gene in *Drosophila*. Two distinct forms of FTZ-F1 had been identified: an early form, which correlated with *ftz* expression during development, and a late form, which was expressed after *ftz* expression was extinguished. Both forms had identical DNA-binding specificities but

apparently differed slightly in their primary structures. These results suggest that the *FTZ-F1* gene in *Drosophila* encodes developmentally specific transcripts that provide alternative functions: a form that is essential for early embryonic development and a form that is expressed later.

A similar situation is found in the mouse. The mouse SF-1 transcript strikingly resembles a cDNA, termed embryonal long terminal repeat-binding protein (ELP), that silenced retroviral gene expression in embryonal carcinoma cells. The two transcripts are essentially identical for 1,100 bp, including the zinc finger DNA-binding domain, but diverge at the 5' and 3' ends. These results indicated that they either derive from distinct but highly related genes or result from alternative splicing of the same gene. Consistent with the latter hypothesis, genomic Southern analyses showed that both the ELP and SF-1 transcripts are encoded by the same gene. This finding suggests that a critical regulator of steroidogenic enzyme gene expression in adult animals is either identical or closely related to a protein that is expressed at high levels in mouse embryonal carcinoma cells. The ELP cDNA presumably corresponds to the early FTZ-F1 protein, whereas the SF-1 cDNA corresponds to late FTZ-F1. This close conservation from *Drosophila* to mouse of developmentally regulated transcripts derived from the *FTZ-F1* gene leads to the intriguing speculation that the late form of FTZ-F1 regulates the enzymes that make ecdysteroids in *Drosophila*.

NGFI-B, Another Nuclear Receptor Protein, May Mediate ACTH Induction of Steroid 21-Hydroxylase

The sequence of the 21-OHase −65 regulatory element closely resembled the canonical response element for nerve growth factor–induced B (NGFI-B), an immediate-early nuclear receptor that is rapidly induced by a variety of growth factors and trophic hormones. The possible role of NGFI-B in regulating steroidogenic enzyme expression was therefore addressed. *In situ* hybridization studies demonstrated high levels of NGFI-B expression in the adrenal cortex, and treatment of mouse Y1 adrenocortical cells with ACTH or cAMP rapidly increased levels of NGFI-B transcripts and protein. Gel mobility shift and DNase I footprinting experiments showed that recombinantly expressed NGFI-B interacts specifically with the 21-OHase −65 element and identified one complex formed by Y1 nuclear extracts that contains NGFI-B. Expression of NGFI-B significantly augmented the activity of the intact 21-OHase promoter and reporter constructs containing the 21-

OHase −65 element. Collectively these results identify a cellular promoter that is regulated by NGFI-B and implicate this nuclear receptor in the transcriptional induction of 21-OHase by ACTH.

The investigations of SF-1 and NGFI-B highlight the critical role of nuclear receptors in the regulation of the steroidogenic enzymes. This role is of significant interest for several reasons. First, the great potential for competition and/or cooperation between various nuclear receptors provides a possible mechanism for implementing the complex regulatory circuits that control steroidogenic enzyme expression. In addition, the link between orphan receptor action and control of steroidogenic enzyme gene expression raises the exciting possibility that metabolic intermediates of steroid products may serve as ligands for these orphan receptors.

Dr. Parker is also Associate Professor of Medicine and Biochemistry at Duke University Medical Center and attending physician at Duke University Medical Center.

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MECHANISM OF ACTION OF POLYPEPTIDE GROWTH FACTORS

Linda J. Pike, Ph.D., *Associate Investigator*

Dr. Pike is interested in the control of cell growth. In particular, her research focuses on the mechanism by which epidermal growth factor (EGF) transmits its signal across the cell membrane. Binding of EGF to the extracellular domain of its cell surface receptor stimulates a protein-tyrosine kinase activity located on the intracellular domain of the receptor. Other biological processes, including phosphatidylinositol (PI) turnover and receptor internalization, are also stimulated by ligand binding. Although the binding of EGF stimulates numerous responses, prolonged treatment of cells with EGF leads to a loss of responsiveness of the cells to the growth factor, a process known as desensitization. The current goals are 1) to understand the regulation of phosphoinositide metabolism and its role in signal transduction and 2) to elucidate the molecular mechanism of EGF receptor desensitization.

Phosphoinositide Metabolism

Phosphatidylinositol 4-kinase. Previous work from the laboratory has shown that EGF stimulates the activity of a PI 4-kinase in A431 cells, a human epidermal carcinoma cell line. The EGF-stimulated PI 4-kinase was purified from A431 cells and shown to be a monomeric protein with a molecular weight of 55,000. PI 4-kinase was also purified from human placenta, and the amino acid sequences of two tryptic peptides from the purified enzyme were determined. Based on the sequence of the longer peptide (40 amino acids), a unique 45-bp oligonucleotide probe was synthesized and used to screen a λgt11 human placenta cDNA library.

Approximately 500,000 independent isolates were screened, and 13 positive clones for PI 4-kinase were identified. The 2.3-kb insert from one of the clones was sequenced and found to encode a protein that contained the sequence of both peptides determined by direct protein sequencing. The predicted protein has a molecular weight of 61,000. The cDNA for the PI 4-kinase was expressed in *Escherichia coli* as a fusion protein with glutathionine *S*-transferase. Assay of *E. coli* lysates demonstrated the presence of increased PI 4-kinase activity

in lysates derived from cells expressing the fusion protein but not in lysates from cells expressing the glutathionine *S*-transferase protein alone. This provides strong evidence that the cloned sequence represents a PI 4-kinase.

The cloned cDNA shows no extensive sequence similarity to any protein or nucleic acid sequences present in computer databases. However, the protein does show limited sequence similarity to two inositol phosphate–binding proteins, inositol 1-polyphosphatase and inositol (1:2) cyclic hydrolase. In addition, the predicted protein contains a putative ATP-binding site of the type found in phosphofructokinase. Consistent with this assignment as the ATP-binding site, the PI 4-kinase is inactivated by treatment with the arginine-specific reagent phenylglyoxal, and inclusion of ATP protects enzyme activity. Neither the PI 4-kinase nor the recently cloned PI 3-kinase shows significant similarity to serine, threonine, or tyrosine protein kinases in this ATP-binding domain, suggesting that the lipid kinases are derived from a different ancestral kinase than are the protein kinases.

The PI 4-kinase can also be inactivated by treatment with fluorosulfonylbenzoyladenosine (FSBA), a reagent that selectively labels ATP-binding sites. The inactivation is concentration and time dependent and is prevented by addition of ATP. Isolation and sequencing of the FSBA-labeled peptide from the PI 4-kinase is currently under way to provide definitive identification of the ATP-binding site of this enzyme. This information will be used to construct altered forms of the PI 4-kinase via site-directed mutagenesis to study structure-function relationships within the enzyme.

Northern blot analysis of RNA derived from a human cell line demonstrated the presence of two hybridizing species of 3.4 and 4.6 kb. When mouse RNA was used, messages of similar size were found to cross-hybridize with the human PI 4-kinase cDNA probe, indicating that the PI 4-kinase is relatively well conserved between these two species. Preliminary evidence suggests that the levels of the 3.4-kb message are increased in monocytes stimulated with phytohemagglutinin, suggesting that PI 4-kinase activity may be regulated at the level of transcription. (The project described above was supported by a grant from the National Institutes of Health.)

Phosphatidylinositol-4-monophosphate (PIP) phosphatase. The reactions in the pathway for the synthesis of the polyphosphoinositides have been presumed to be reversible; however, the enzymes responsible for the dephosphorylation of PIP and PIP$_2$ (phosphatidylinositol 4,5-bisphosphate) have

not been extensively characterized. A PIP phosphatase from rat brain has been extensively purified in Dr. Pike's laboratory. Physical and biochemical studies suggest that it is a glycoprotein with a molecular weight of 70,000–90,000. Additional work will focus on the complete purification of this enzyme as well as the generation of antibodies and protein sequence necessary for cloning of this phosphatase.

Desensitization of the EGF Receptor

Previous work in Dr. Pike's laboratory has shown that treatment of A431 cells with high concentrations of EGF reduces their ability to internalize ^{125}I-EGF. Additional studies suggested that several other EGF-stimulated responses are also desensitized after treatment of A431 cells with EGF. These include EGF-stimulated PI turnover and EGF-stimulated tyrosine protein kinase activity. Desensitization was also associated with an impairment in the ability of EGF to induce receptor dimer formation. The desensitization was homologous, or agonist specific, in nature and did not appear to involve protein kinase C–mediated events.

In several systems in which receptor desensitization has been studied, the underlying molecular basis for desensitization has been shown to involve phosphorylation of the receptor protein. Therefore studies of the phosphorylation state of desensitized receptors were undertaken. These studies identified a serine-containing phosphopeptide in the EGF receptor, the presence of which correlated with the condition of desensitization. The identity of the peptide was determined by both direct amino acid sequencing and radiosequencing of *in vivo* ^{32}P-labeled peptide.

A peptide was synthesized based on the sequence surrounding this site of phosphorylation of the EGF receptor. A kinase activity capable of phosphorylating the synthetic peptide was identified in A431 cell cytosol. This activity was shown to be stimulated acutely following treatment of the cells with EGF. Partial purification and characterization of the kinase suggested that it had a molecular weight of ~90,000. The kinase was identified as p34^{cdc2}, based in part on this molecular weight estimation combined with the ability of anti-p34^{cdc2} antibodies to immunoprecipitate peptide kinase activity.

Incubation of purified EGF receptors with p34^{cdc2} demonstrated that the serine kinase directly phosphorylated the EGF receptor. Phosphorylation of the EGF receptor by p34^{cdc2} led to a reduction in the tyrosine kinase activity of the receptor. Future experiments will examine the effect of this phosphorylation on the ability of EGF to induce receptor

dimer formation to determine whether p34^{cdc2} is responsible for desensitizing the EGF receptor. In addition, studies are in progress to determine the effect on EGF receptor function of altering the site of p34^{cdc2} phosphorylation.

Dr. Pike is also Associate Professor of Biochem-

MOLECULAR AND CELL BIOLOGY OF BLOOD COAGULATION

J. Evan Sadler, M.D., Ph.D., *Associate Investigator*

Dr. Sadler is interested in the structure, function, and regulation of proteins that are required for hemostasis. In particular, he studies how cells that contact the blood maintain a balance between stimulating and inhibiting blood coagulation and how this balance is disrupted in human diseases. Dr. Sadler concentrates on two areas: 1) von Willebrand factor and von Willebrand disease and 2) the regulation of blood coagulation by endothelial cells.

Molecular Biology of von Willebrand Factor and von Willebrand Disease

von Willebrand factor (vWF) is a multimeric plasma protein that is synthesized by vascular endothelial cells. It is required for platelet adhesion to sites of injury and for normal survival of factor VIII in the circulation. von Willebrand disease (vWD) is the most common inherited bleeding disorder of humans and is phenotypically heterogeneous.

vWD is divided into three categories. Type I, the most common form, is characterized by partial quantitative deficiency of vWF and is inherited as a dominant disorder. Type II is characterized by qualitative deficiency; many subtypes are recognized that differ in mode of inheritance, clinical severity, and properties of the dysfunctional protein. Type III is an autosomal-recessive, severe disease with virtual absence of vWF. Knowledge of the molecular defects in vWD will illuminate structure-function relationships of vWF and may lead to improved therapy.

von Willebrand disease type IIA. vWF mediates platelet adhesion by binding to platelet glycoprotein (GP) Ib. vWD type IIA is characterized by decreased binding to GPIb and by the absence from plasma of high-molecular-weight vWF multimers. Mutations in this subtype cluster within the A2 domain of the mature vWF subunit that is encoded by exon 28. Dr. Sadler and his colleagues found three missense mutations in this exon that result in the amino acid substitutions Arg(834) → Trp,

Gly(742) → Glu, and Ser(743) → Leu. The first mutation occurred independently in three unrelated families; each of the latter mutations was found in one family. The high frequency of identical independent mutations suggests that precise diagnosis of vWD type IIA may be possible in a majority of patients by using simple DNA screening assays.

von Willebrand disease Normandy. In vWD Normandy, a rare type II variant, the mutant vWF appears structurally and functionally normal except that it does not bind to blood coagulation factor VIII. This interaction is required for normal survival of factor VIII in the circulation; consequently, vWD Normandy can present as apparent hemophilia A but with autosomal-recessive rather than X chromosome–linked inheritance. Missense mutations in or near the factor VIII–binding site on vWF have been identified in several families with vWD Normandy. Patients from one family were found to be homozygous for a C → T transition in a CG dinucleotide, converting Arg(53) to Trp. The corresponding recombinant mutant vWF had the same defect in factor VIII binding as the patients' plasma vWF, confirming that the Arg(53) → Trp mutation causes vWD Normandy. These results suggest that the disulfide loop containing Arg(53) may bind factor VIII; this region of vWF was not previously known to be involved in this interaction.

Regulation of Blood Coagulation

The vascular endothelium normally inhibits blood-clotting reactions. The anticoagulant properties of endothelium are due in part to the expression of thrombomodulin, a cell surface receptor that binds the serine protease thrombin and alters its substrate specificity.

Thrombin is a multifunctional protease that has both procoagulant and anticoagulant activities. As a procoagulant enzyme, thrombin clots fibrinogen, activates clotting factors V and VIII, and activates

platelets. On binding to thrombomodulin, however, the procoagulant activities of thrombin are reduced, and its ability to activate protein C, a serine protease zymogen, is greatly increased. Activated protein C degrades clotting factors Va and VIIIa. Thus the formation of the thrombin-thrombomodulin complex converts thrombin from a procoagulant into an anticoagulant enzyme, and the normal balance between these opposing activities is critical to the regulation of hemostasis.

Inhibition of thrombomodulin expression by the thrombogenic agent homocysteine. Homocysteinemia caused by cystathionine β-synthase deficiency is associated with thrombosis and atherosclerosis, but the mechanisms responsible for this association are unknown. Studies in cultured human endothelial cells, or CV-1 cells expressing recombinant human thrombomodulin, suggest that homocysteine can reduce thrombomodulin cell surface expression. Homocysteine produced slight increases in thrombomodulin mRNA and thrombomodulin synthesis without affecting cell viability. The newly synthesized thrombomodulin was not transported to the cell surface but was retained in the endoplasmic reticulum. By altering the expression of hemostatic proteins in endothelial cells, homocysteine may contribute to the thrombosis in patients with cystathionine β-synthase deficiency.

Thrombomodulin structure-function relationships. Thrombomodulin contains an amino-terminal extracellular domain, one transmembrane domain, and a short cytoplasmic tail. The extracellular domain is composed of a lectin-like domain, six tandemly repeated epidermal growth factor (EGF)-like domains, and an O-glycosylated Ser/Thr-rich domain. EGF-like domains 5 and 6 contain a major thrombin-binding site. Thrombin-binding sequences were further localized by employing synthetic peptides derived from thrombomodulin to inhibit thrombin binding. Each EGF-like domain contains ~40 amino acids that form three disulfide loops. The most active peptide inhibitors corresponded to the third loop of the fifth EGF-like domain and to parts of the second and third loops of the sixth EGF-like domain. Replacement of the Ser/Thr-rich domain with a segment devoid of glycosylation sites did not affect the cofactor function of thrombomodulin, whereas substitution of this domain with segments of decreasing length progressively decreased both cofactor activity and thrombin-binding affinity. Thus the Ser/Thr-rich domain is required to position the thrombin-binding site optimally above the membrane surface, and O-linked glycosylation is not required for cofactor activity.

Ligand specificity of human thrombomodulin. Thrombomodulin has been proposed to bind proteases other than thrombin and thereby to regulate blood coagulation by several independent mechanisms. Both bovine factor Xa and bovine meizothrombin, an intermediate product of prothrombin activation, have been reported to catalyze thrombomodulin-dependent activation of protein C. To examine the ligand specificity of human thrombomodulin, equilibrium binding assays were performed with human thrombin, thrombin S205A (wherein the active-site serine is replaced by alanine), meizothrombin S205A, and human factor Xa. Human meizothrombin did not compete with thrombin for binding to recombinant human thrombomodulin, nor did meizothrombin bind directly to thrombomodulin. Similarly, human factor Xa did not bind to thrombomodulin, nor did it catalyze the thrombomodulin-dependent activation of protein C. These results suggest that, in contrast to results reported for the bovine proteins, human meizothrombin and factor Xa are unlikely to be important thrombomodulin-dependent activators of protein C and that thrombin is the physiological protease ligand for human thrombomodulin.

These studies will improve understanding of how endothelial cell proteins may participate in inflammation and hemostasis. Such knowledge may suggest new strategies for the treatment of patients with bleeding or thrombosis. (The projects described in this section are supported by a grant from the National Institutes of Health.)

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MECHANISM OF INTRACELLULAR PROTEIN TRANSPORT

Randy W. Schekman, Ph.D., *Investigator*

Research in Dr. Schekman's laboratory is focused on the molecular mechanism of protein traffic in the secretory pathway. A combined genetic and biochemical approach was developed to study this pathway in *Saccharomyces cerevisiae*. The isolation of a large number of mutations that define the secretory pathway and the characterization of the affected gene products have demonstrated that the pathway and the molecular machinery of protein transport are highly conserved among eukaryotes. A cell-free reaction that reproduces roughly the first half of the secretory pathway is now being used to isolate functional forms of the cytosolic and membrane-bound proteins of the secretory machinery.

Vesicle-mediated Protein Transport from the Endoplasmic Reticulum

More than 20 *SEC* genes are required for the formation, targeting, and fusion of small vesicles that carry proteins from the endoplasmic reticulum (ER) to the Golgi apparatus. Cytologic evaluation of mutant cells has allowed the assignment of roles in vesicle budding and targeting to seven of these genes. A more thorough analysis of the role of the complete set of gene products involved in this limb of the secretory pathway has become possible through the development of a cell-free system that reproduces the transport-dependent glycosylation of *in vitro* synthesized α-factor precursor. This reaction is quantified by precipitation of the radioactive product, using antibodies directed against carbohydrate epitopes that are covalently attached to the glycoprotein substrate in the Golgi apparatus. Transport is dependent on ATP, GTP hydrolysis, cytosol, and intact membranes. The radioactive marker is transferred between physically separable organelles (ER and Golgi) via an intermediate vesicle that has distinctive properties. Transport is temperature sensitive in extracts of certain thermosensitive *sec* mutant strains and is inhibited by antibodies directed against recombinant or native forms of Sec proteins.

A simplified assay has been developed that measures only the vesicle budding reaction. The α-factor precursor, trapped within the ER, sediments rapidly, whereas when vesicle transport begins the precursor appears within slowly sedimenting vesicles. Budding can thus be followed simply by differential centrifugation of lysates. This reaction has allowed the isolation of vesicles produced *in vitro*. Surprisingly, vesicles are devoid of three different Sec proteins required for budding (Sec12p, Sar1p, Sec13p) but contain Sec proteins that are required for targeting (Sec22p, Ypt1p).

Proteins that are required for vesicle budding have been purified by several strategies. A complex that includes two Sec proteins (Sec23p and Sec24p) has been purified using biochemical complementation of a *sec23* mutant lysate as a functional assay. The small GTP-binding protein Sar1p has been shown to be removed selectively from the cytosol fraction in cells that overproduce the integral membrane glycoprotein Sec12p, which is also required for vesicle budding. Complementation of such a depleted cytosol fraction provides a functional assay that has allowed purification of Sar1p. Because the budding reaction requires intact membranes, it has not been possible to isolate a functional form of Sec12p from detergent-solubilized membranes. However, deletion of the carboxyl-terminal luminal domain and the single membrane anchor segment of Sec12p releases an amino-terminal cytosolic do-

main that is a potent inhibitor of budding. This inhibitory effect is reproduced with pure Sec12p cytosolic fragment protein.

Finally, the last known cytosolic component, Sec13p, has been purified using a strategy that involves a functional *SEC13*–dihydrofolate reductase (DHFR) fusion gene. Cytosol from a yeast strain that harbors the hybrid gene as its sole copy of *SEC13* is specifically depleted of the DHFR hybrid protein by adsorption to a methotrexate affinity column. Depleted cytosol plus a methotrexate-eluted fraction restores vesicle budding to a urea-washed membrane fraction. Pure Sec13p isolates as a large complex and includes a new 150-kDa protein that is also required for budding. Mixture of pure Sec23/24p, Sar1p, and Sec13/p150 restores vesicle budding to urea-washed membranes. Hence Dr. Schekman and his co-workers are now in a position to examine the mechanics of the budding reaction with a set of pure proteins and an isolated membrane fraction.

The first important clue to regulation of the budding event has come with the detection of a cycle of GTP hydrolysis mediated by pure Sec proteins. Sar1p performs slow GTP hydrolysis and nucleotide exchange that require detergent or phospholipid. GTP hydrolysis by Sar1p is stimulated 10-fold by the Sec23p subunit of the 23/24p complex. GTP-GDP nucleotide exchange on Sar1p is stimulated fivefold by the cytosolic domain of Sec12p. These requirements confirm and extend the observation that a nonhydrolyzable analogue of GTP (GTPγS) retards vesicle budding. The signal that triggers nucleotide exchange, the target of Sar1p-GTP, and the mechanistic coupling to vesicle budding are open questions.

Protein Translocation from the Cytosol Into the Endoplasmic Reticulum

With support by a grant from the National Institutes of Health, Dr. Schekman's laboratory has identified four *SEC* genes (*SEC61*, *SEC62*, *SEC63*, and *SEC65*) that are required for polypeptide translocation into the ER. Three of the gene products are integral proteins localized to the ER membrane. Sec61p is very hydrophobic, with from five to eight potential membrane-spanning domains. Sec62p and Sec63p each have two membrane-spanning domains and significant soluble domains that are oriented toward the cytosol. Antibodies directed against the cytosolic domains of Sec62p or Sec63p precipitate a complex of five polypeptides from detergent-solubilized membrane fractions. In addition to the three identified Sec membrane proteins, the complex includes two new proteins: a 31.5-kDa glyco-

protein and a 23-kDa nonglycosylated polypeptide. Clones of both genes have been obtained.

A firm connection between the three Sec membrane proteins and the translocation event has been established by chemical crosslinking of a translocating polypeptide to the Sec protein complex. A translocation substrate protein, yeast α-factor precursor, has been altered to contain a carboxyl-terminal cysteine residue, which allows covalent coupling via a heterobifunctional crosslinking reagent to the egg white protein, avidin. When diluted out of a urea solution, the α-factor precursor portion of the conjugate penetrates the ER membrane but becomes stuck because avidin, which is not denatured by the urea treatment, remains on the cytosolic face of the ER. Addition of a different cleavable crosslinking agent to the inhibited membranes produces a macromolecular complex that may be isolated from a detergent-solubilized fraction by precipitation with Sec61p antibody. Formation of the complex requires hydrolyzable ATP and is blocked when membranes isolated from the translocation-defective mutants are used. Thus the Sec protein complex makes intimate contact with a translocating polypeptide and may represent the elusive translocation pore or channel that has been sought for more than 20 years.

Current effort is devoted to the isolation of a functional translocation complex. Yeast membranes are solubilized with a mixture of octylglucoside and phospholipid and dialyzed to remove the detergent, and proteins are integrated into preformed phospholipid vesicles by sonication. Reconstituted vesicles are competent for translocation and signal peptide processing of α-factor precursor in a reaction that is dependent on ATP and stimulated by a crude cytosol fraction. Fractionation of the detergent-soluble material has allowed the isolation of a functional complex that includes Sec63p, the luminal hsc70 isozyme BiP, and the two new proteins that were identified in crosslinking studies (p23 and gp31.5). The participation of BiP appears to be specific: cytosolic hsc70 does not replace the requirement for BiP in reconstituted proteoliposomes formed from BiP mutant membranes. An effort to resolve completely the membrane components required to reconstitute translocation is under way.

Dr. Schekman is also Professor of Biochemistry and Molecular Biology in the Department of Cell and Molecular Biology, University of California, Berkeley, and Adjunct Professor of Biochemistry and Biophysics at the University of California, San Francisco.

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STUDIES IN BACTERIAL PATHOGENESIS

Gary K. Schoolnik, M.D., *Associate Investigator*

Many bacterial species that are pathogenic for humans exist in a variety of environmental habitats that differ greatly with respect to temperature, the availability of nutrients, and the concentrations of simple chemical compounds including oxygen, iron, calcium, and phosphate. It now seems likely, based on studies from several laboratories, that physicochemical parameters of this kind serve as markers of a particular habitat, leading to the induction of bacterial genes that form an adaptive response.

Particularly useful examples of this phenomenon are the enteric gram-negative bacteria, especially pathogenic *Escherichia coli*, the *Salmonellae*, and *Vibrio cholerae* (the agent of Asiatic cholera). The principal biomass of each of these may not be in the intestines of animals and humans but rather in sewage, water, and soil, where they can exist as free-living entities or in association with plants and other simple organisms. The genetic basis for their capacity to survive in these extraintestinal niches has not been much studied by medical microbiologists but was probably acquired early in their evolution—prior to the arrival of vertebrates—and arguably underlies their capacity to persist as successful biological entities in nature.

On their transfer from one of these habitats to the human intestine through the ingestion of contaminated food or water, a rapid and dramatic change in bacterial metabolism and gene expression occurs, and new proteins are elaborated that allow the organism to attach to mucous membranes, secrete potent enterotoxins, or enter epithelial cells. What role the intestinal phase of their life cycles might play in the ecology of these organisms and the origin of the genes that subserve this phase are largely unanswered questions. Possibly, as a result of significantly increased replication rates in the intestine, the organisms are shed in large numbers in the feces,

thus locally boosting their biomass in natural habitats above levels that are critical for their continued survival. From a medical and public health perspective, the genetic mechanisms that underlie both the intestinal and the environmental phases of their lifestyles are of interest, because an understanding of the former may lead to new vaccines, while an appreciation of the latter could lead to new strategies for their eradication from the biosphere.

In the preceding year, Dr. Schoolnik's laboratory has studied biochemical and genetic events in enteropathogenic *E. coli* (EPEC) that appear to transpire within minutes of the organism's infection of the small intestine; the evolution of virulence genes of *V. cholerae* was also examined.

Adaptation of EPEC to an Intraintestinal Life-Style

EPEC are a significant cause of childhood diarrhea, affecting millions of children each year. The principal reservoir in nature of EPEC is contaminated water, where it grows as free-living, isolated bacteria. However, small bowel biopsies of children infected with EPEC show discrete colonies of the organism attached to intestinal mucous membranes. Thus on entering the intestinal environment, EPEC adopt a colonial mode of growth. This process has been studied experimentally and shown to occur within 60 min of the organism's contact with cultured human cells. Ultrastructural studies show that the organism produces new surface structures during this transition that appear to mediate colony formation.

These structures are composed of tens to hundreds of small filaments (termed bundle-forming pili [BFP]) that emanate from the surface of each bacterium. The filaments of adjacent organisms become entwined to form bundles of filaments within which are embedded the bacteria of the colony. Each BFP is composed of a principal, repeating 19.5-kDa subunit that is structurally related to other members of the type 4 family of pilins, a widely disseminated gene found in pathogenic *Neisseriae, Pseudomonads, Vibrios, Bacteroides*, and *Moraxellae*. The structural gene of the BFP subunit, *bfp*, is located on a 60-MDa plasmid, known to be important for virulence and for the colony-forming phenotype. Other genes carried by this plasmid are required for the biogenesis of mature BFP, including a gene that directs the cleavage of the BFP precursor at an unusual signal peptidase recognition site; another gene appears to be required for the assembly of the subunits into a filamentous polymer. The specific physicochemical signal within the intestinal milieu and how this signal effects induction of the BFP operon are under investigation.

Evolutionary Source of *V. cholerae* Virulence Genes

Most cholera epidemics originate in the Ganges delta of India and Bangladesh, an esturine region where the salinity and the availability of other solutes varies according to the season. The cholera case rate also is seasonally determined, and there is now compelling evidence that in the interepidemic period, *V. cholerae* persists in the environment as a marine organism. To determine if this capacity may have originated with primitive nonpathogenic marine vibrios, Dr. Schoolnik and his colleagues sought homologues of two *V. cholerae* virulence genes in *V. fisheri*, a bioluminescent commensal of the light organs of certain fish and squid. The epithelia of these light organs and their interaction with *V. fisheri* resemble histologically the interaction of *V. cholerae* with mammalian intestinal surfaces. By using *ctxA* and *ctxB* of *V. cholerae* as probes, low-stringency hybridization experiments revealed homologues of each in a *V. fisheri* genomic library. Similarly, a homologue of the *V. cholerae* ToxR protein, which serves to regulate the expression of cholera toxin in response to changes in pH and osmolality, was immunologically identified in *V. fisheri* through the use of ToxR-specific antisera. The enzymatically active fragment of cholera toxin (CtxA) ADP-ribosylates the regulatory protein–guanosine triphosphate complex of adenyl cyclase leading to the production of cAMP.

Consistent with the DNA hybridization experiments described above, *V. fisheri* lysates were found to have ADP-ribosylating activity, and the organism was able to subsist on cAMP as its sole carbon source. These findings indicate that the cholera toxin ancestral genes in *V. fisheri* may have been regulated by varying degrees of salinity in the marine environment, where they originally functioned to support the commensalism of this species for its nonmammalian hosts through the production of cAMP. If so, the ToxR/cholera toxin precursors, once installed in *V. cholerae*, may have acquired a new role as the principal virulence determinants of the organism, while retaining their original ability to permit its persistence in the brackish water of estuaries between epidemics of human disease.

Dr. Schoolnik is also Associate Professor of Medicine and of Microbiology and Immunology at Stanford University School of Medicine.

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ADENOVIRUS GENE EXPRESSION AND ONCOGENESIS

THOMAS E. SHENK, PH.D., *Investigator*

Transcriptional Activation by Adenovirus E1A Proteins

The *E1A* gene is the first adenovirus gene to be expressed when the viral chromosome reaches the nucleus of a newly infected cell. The products of the *E1A* gene then activate transcription of all remaining early viral genes and induce expression of the *E1A* gene itself. The mechanism by which E1A proteins activate transcription is only partially understood. However, several cellular transcription factors that play roles in the process have been identified.

One of these cellular factors is YY1. A binding site for YY1 was first identified in the P5 promoter of adeno-associated virus, a defective parvovirus that depends on a variety of adenovirus gene products—including E1A proteins—for its replication. The YY1-binding site repressed transcription mediated by the P5 promoter as well as heterologous promoters, and the adenovirus E1A protein relieved the repression and further activated transcription through the sequence motif.

To study the YY1 factor better, Dr. Shenk and his colleagues cloned its cDNA with a probe DNA corresponding to an amino acid sequence determined from purified protein. YY1 is a 414–amino acid polypeptide that contains four C_2H_2-type zinc fingers, and the sequences of these motifs are related to those of the GLI-Krüppel family of DNA-binding proteins.

The binding activity of the cloned YY1 protein was altered by fusing it to the DNA-binding domain of the yeast GAL4 protein. By redirecting the factor to a new binding site, it was possible to study the activity of the fusion protein in cells that contain high endogenous levels of YY1. The fusion protein repressed transcription of genes containing a GAL4 DNA-binding site, and the repression was relieved by E1A proteins. Thus the cloned protein displays the same biological properties as the activity in HeLa cells that interacts with YY1-binding sites. Furthermore, since the viral E1A protein would not be expected to cause the YY1 fusion protein to detach from the GAL4 DNA-binding site, it appears likely that YY1 remains bound to the control region but is somehow altered in the presence of E1A proteins so that transcription is not repressed.

The domain of the YY1 protein that mediates repression was mapped by fusing fragments of the protein to the GAL4 DNA-binding domain and testing the ability of the fusion proteins to repress promoters that contain GAL4 DNA-binding sites. The carboxyl-terminal, zinc finger region of the YY1 protein contained the repression activity. This domain of YY1 is also responsible for its ability to bind to YY1 DNA recognition sites. It is not clear whether the zinc fingers are directly involved in the repression activity, and, if they are, whether DNA-binding and repression functions are mediated by the same amino acid sequence. Single amino acid changes are

being introduced into the zinc finger domain to address this issue.

E1A appears to bind directly to YY1 to modulate its activity. When E1A and YY1 proteins are mixed, they form a complex that can be detected by sedimentation in a sucrose gradient. The sedimentation coefficient of the complex suggests that it is a heterodimer. An E1A-YY1 interaction can also be detected when [^{32}P]-labeled E1A protein is used to probe a protein blot in which YY1 protein has been subjected to electrophoresis and transferred to a nitrocellulose membrane. This experiment argues that the interaction is direct, because both proteins were produced in bacterial cells, eliminating the possibility that an additional eukaryotic protein was present and serving to bridge between E1A and YY1.

Segments of the YY1 protein were tested for their ability to interact with the E1A protein. The E1A-YY1 interaction maps to the central third of the YY1 protein, at a site distinct from the carboxyl-terminal repression domain. Experiments are in progress to search for cellular proteins that might bind to YY1 within its repression and E1A interaction domains.

Role for an Adenovirus E4 Protein in Rat Mammary Oncogenesis

All adenoviruses that have been tested are able to transform established rodent cell lines oncogenically, and some adenoviruses can induce sarcomatous tumors at the site of inoculation into rodents. Ad9 appears to be unique among the adenoviruses in that it induces exclusively estrogen-dependent mammary tumors in female rats. These tumors include mammary fibroadenomas, which are common benign breast tumors of women, as well as phyllode-like tumors and sarcomas.

Examination of the viral genes expressed in a variety of Ad9-induced rat mammary tumors revealed that E1A but not E1B mRNA was present. The tumors also all contained mRNAs encoded by the Ad9 *E4* gene. This was in contrast to sarcomas induced by other adenovirus serotypes that invariably contain mRNAs encoded by both *E1A* and *E1B* oncogenes but only occasionally express E4 mRNAs.

By constructing recombinant viruses between Ad9 and Ad26 (a related adenovirus that does not induce mammary tumors), it was possible to demonstrate that the Ad9 *E4* gene is required to produce mammary tumors. This gene is also able to induce the formation of transformed foci in an established rat cell line, suggesting it plays a direct role in oncogenesis. This demonstrates for the first time that an adenovirus gene outside of the classic E1A/E1B-transforming region can influence *in vivo* oncogenesis. Work is in progress to identify the specific Ad9 E4 protein involved (*E4* encodes at least six polypeptides) and to elucidate the mechanism by which it contributes to mammary oncogenesis. (This project was supported by grants from the National Cancer Institute, National Institutes of Health.)

Dr. Shenk is also James A. Elkins, Jr., Professor of Molecular Biology at Princeton University and Adjunct Professor of Biochemistry at the University of Medicine and Dentistry of New Jersey, Robert Wood Johnson Medical School.

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HEMATOPOIETIC GROWTH CONTROL AND ONCOGENESIS

Charles J. Sherr, M.D., Ph.D., *Investigator*

Colony-stimulating factor 1 (CSF-1) regulates the proliferation, differentiation, and survival of mononuclear phagocytes. Macrophages deprived of CSF-1 undergo growth arrest early in the first gap phase (G_1) of the cell cycle and ultimately die. However, if transiently starved cells are restimulated with the growth factor, they progress synchronously through G_1 and begin to replicate their chromosomal DNA (S phase) 10 h later. CSF-1 stimulation is required throughout G_1 to ensure cellular DNA synthesis, but once cells commit to enter S phase, they can complete cell division without the growth factor. CSF-1 binds to membrane-spanning cell surface receptors encoded by the *FMS* proto-oncogene, and the ligand-induced activation of the CSF-1 receptor (CSF-1R) protein-tyrosine kinase (PTK) triggers signal transduction through multiple second messenger pathways. The latter signals act in turn to govern the transcription of CSF-1–responsive genes, thereby determining the magnitude and specificity of the biologic response and, ultimately, the decision to enter S phase.

The central goals of Dr. Sherr's laboratory are to elucidate mechanisms by which CSF-1 governs proliferative and differentiative decisions throughout the G_1 interval and to understand how perturbations that deregulate elements of CSF-1R signal transduction pathways contribute to tumorigenesis.

Independent Mutations in CSF-1R Convert It to an Oncoprotein

Mutations in the CSF-1R extracellular domain that mimic a conformational change induced by ligand binding constitutively induce receptor PTK activity and provide sustained signals for cell growth. To determine whether multiple sites for such "activating mutations" might exist, Dr. Sherr and his colleagues subjected portions of *FMS* cDNA-encoding regions of the receptor extracellular domain to chemical mutagenesis and reinserted them into a retroviral expression plasmid to generate libraries of *FMS* genes containing random mutations confined to predetermined target cassettes. Transfection of these plasmids into fibroblasts yielded morphologically transformed cells from which retroviruses could be rescued. Amplification by polymerase chain reaction of target cassettes from unique integrated proviruses and nucleotide sequencing revealed that activating mutations could occur at many *FMS* codons and that certain residues were

"hot spots." Some of the latter codons in CSF-1R are conserved in position and context in the evolutionarily related receptors for platelet-derived growth factor (PDGF) and in the receptor for *Steel* factor (SLF or *KIT* ligand), suggesting that analogous mutations might potentially convert these receptors to ligand-independent oncoproteins.

CSF-1R Signals through Multiple Pathways

Ligand binding leads to CSF-1R dimerization, activation of receptor PTK activity, and cross-phosphorylation of aggregated receptor subunits on tyrosine. Sites of receptor tyrosine phosphorylation contribute to form recognition motifs for certain cytoplasmic effector molecules, whose binding to the receptor and/or phosphorylation on tyrosine mediate downstream biochemical responses to the growth factor. Therefore mutations that selectively eliminate sites of tyrosine phosphorylation within CSF-1R can disrupt its interactions with particular effector proteins and abrogate signaling through specific downstream pathways. Dr. Sheila Shurtleff previously found that mutation of one such phosphorylation site (Tyr-809) in the cytoplasmic domain of CSF-1R had no effect on receptor PTK activity, binding to phosphatidylinositol 3-kinase, or the induction of several immediate-early response genes, including c-*fos*, *jun*B, c-*jun*, or c-*ets*2, all of which encode transcription factors. However, the c-*myc* response to CSF-1 was significantly impaired, and cells bearing the mutant receptor were unable to proliferate in response to the growth factor. Enforced expression of an exogenous c-*myc* gene rescued the cells' ability to grow in response to CSF-1.

These results reveal a bifurcation of CSF-1 signal transduction pathways that target *fos/jun* on the one hand and c-*myc* on the other and demonstrate that the latter gene is required for mitogenicity. Dr. Sherr and his colleagues are now using cells bearing this CSF-1R mutant as a genetic "trap" to screen for genes that act downstream of the receptor in controlling the c-*myc* response.

CSF-1 Regulates the Expression of G_1 Cyclins

Last year, Dr. Hitoshi Matsushime cloned novel D-type cyclin genes that are induced by CSF-1 during the mid-to-late G_1 interval. By analogy to B-type cyclin, which regulates the activity of the cell division cycle kinase $p34^{cdc2}$ to control mitotic entry

and exit in all eukaryotic cells, G_1 cyclins are presumed to function as regulatory subunits of other cyclin-dependent kinases that instead govern G_1 progression and entry into S phase. In mammalian cells, such genes might also control differentiative decisions made during the G_1 interval that are accompanied by transient cell cycle arrest.

Cloning of murine and human D-type cyclins has now revealed the presence of three highly conserved genes that map to different chromosomes, have similar intron-exon organizations, and are independently regulated by growth factors in most, if not all, mammalian cells. For example, following restimulation of growth factor–deprived macrophages with CSF-1, cyclin D1 induction precedes that of cyclin D2, whereas cyclin D3 is not expressed. Cyclin D1 mRNA and protein levels remain elevated in the proliferating cells as long as CSF-1 is present, but the expression of cyclin D2 is periodic and is maximal at the G_1/S transition. In contrast, in peripheral blood T lymphocytes stimulated with mitogens to enter the cell cycle, cyclins D2 and D3 are expressed, but cyclin D1 is not, and expression of cyclin D2 temporally precedes that of cyclin D3. All of the cyclin D proteins have short half-lives, and premature withdrawal of CSF-1 during the G_1 interval leads to their rapid degradation, correlating with the subsequent failure of cells to enter S phase.

During G_1 the cyclin D proteins associate with $p34^{cdc2}$-like polypeptides and are themselves phosphorylated. A future challenge will be to identify these cyclin-dependent kinases and their key physiologic substrates. Possible substrates for complexes between D-type cyclins and their catalytic subunits include the retinoblastoma gene (*Rb-1*) product, whose phosphorylation in late G_1 appears to be required for S-phase entry. The different D-type cyclins possibly interact with more than one such kinase, further suggesting that their functions are unlikely to be redundant. Perturbations of either cy-

clin D1 or D2 gene expression have now been demonstrated to result from chromosomal translocations, inversions, retroviral insertions, and gene amplification in lymphomas, thymomas, parathyroid adenomas, squamous cell carcinomas, and in primary adenocarcinomas of the breast, implying that their deregulation can contribute to neoplasia.

Dr. Sherr is also a member of the Department of Tumor Cell Biology at St. Jude Children's Research Hospital and Adjunct Professor of Biochemistry at the University of Tennessee College of Medicine, Memphis.

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DEVELOPMENTAL CHANGES IN THE *DROSOPHILA* GENOME DURING OOGENESIS

ALLAN C. SPRADLING, PH.D., *Investigator*

The genetic information for development is stored within the DNA backbone making up each chromosome, primarily as encoded RNA and protein sequences. Other DNA regions mediate expression of only the appropriate genes in each cell of the developing organism. At least occasionally this in-

formation involves changes in the relative copy number (gene amplification) or structure (gene rearrangement) of the DNA sequences themselves within specific developing cells, as part of their program of differentiation. Dr. Spradling's laboratory is interested in the molecular mechanisms controlling

genomic modifications during development and in their functional significance.

Dp1187 and the Y Chromosomes: Genomic Changes During Development

Dr. Spradling's studies have primarily utilized *Dp1187*, the smallest known functional chromosome in any multicellular eukaryote. A 150- to 220-kb block (1 kb = 1,000 DNA base pairs) of *Dp1187* "euchromatin" encodes nine genes, while the remaining 1,100 kb is classed as heterochromatin, a little-understood but common component of eukaryotic genomes frequently associated with centromeres and telomeres. Heterochromatic regions are folded differently than euchromatic ones, consist mostly of repeated DNA sequences and transposons, and undergo copy number changes during *Drosophila* development.

The *Drosophila* Y chromosome provides a second system for these studies. It encodes a nucleolus and only six genes, despite being 40 times larger than *Dp1187*. The only functional requirement for these genes is during spermatogenesis, and Y-linked sequences are virtually lost in many other differentiated cells during development.

The most dramatic changes detected so far are in the size of *Dp1187* molecules. The chromosome migrated on pulsed-field gel electrophoresis of DNA from embryos as 1,300 kb in length, but in the ovary two new bands of only 680 kb and 400 kb were observed. These altered molecules derived from the two major ovarian cell types, the nurse and follicle cells. Smaller, more heterogeneous *Dp1187* molecules were also found in these cells with a frequency that correlated with the variegated expression of a marker gene on the chromosome. Dr. Spradling and his colleagues interpreted these observations as supporting their previous proposal that a DNA-elimination-like process occurs during the normal development of *Drosophila* nurse and/or follicle cells. (These studies were also supported by a grant from the National Institutes of Health.)

Genetic Regulation of Oogenesis

Dr. Spradling's group has continued to study several aspects of *Drosophila* oogenesis that may be related to the changes described above. The cells that will contribute to each new egg derive from both germline and somatic stem cells. The number, location, and stem cell character of these cells are being investigated. Laser ablation studies verified the presence of a small number of germline stem cells in the anterior part of each ovariole. Three different groups of somatic cells were identified that adjoin these cells as candidate regulators of their function. Three genes, *fs(1)Yb*, *ovarette*, and *piwi*, have been identified and cloned that appear to be required for normal germline stem cell function. Mutations in any one of these genes caused all the germline cells in the ovary to differentiate. One of the genes, *piwi*, has a similar effect on male germline stem cells in the testis.

Several later steps in oogenesis are also being studied. Nurse cells produce large amounts of ribosomes within an unusually large nucleolus and transport them to the oocyte. Several genes have been identified in which nurse cells fail to function properly and degenerate. The possible role of juvenile hormone in regulating egg development has also been addressed by isolating several new genes that block development near the onset of yolk uptake, a checkpoint thought to be controlled by this hormone.

Drosophila Genome Resource

Large-scale genetic screens for mutations induced by single P elements have proven of great value over the past five years. A collection of 1,800 single P-element–induced lethal mutations generated here and in the laboratories of Drs. Gerald Rubin (HHMI, University of California, Berkeley), Matthew Scott (Stanford University), and Lily and Yuh Nung Jan (HHMI, University of California, San Francisco) have recently been incorporated into a genome project designed to facilitate molecular and genetic studies of *Drosophila*. In collaboration with Dr. Rubin, localization and complementation studies will whittle the collection to ~1,100 strains, each of which defines a different vital locus. Sequences flanking each insertion site will be physically mapped by collaborators at Lawrence Berkeley Laboratories (Michael Palazzolo and Christopher Martin) and at Harvard (Dr. Daniel Hartl) with support from the National Center for Human Genome Research, National Institutes of Health. Information developed by the project will be distributed on-line, and both clones and insertion strains will be furnished to the research community.

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MOLECULAR AND CELL BIOLOGY OF THE ISLETS OF LANGERHANS

Donald F. Steiner, M.D., *Senior Investigator*

During the past year progress has been made in studies on the mechanisms of conversion of prohormones to their biologically active forms in the islets of Langerhans and other neuroendocrine tissues. PC2 and PC3, two novel subtilisin-related proteases identified last year, have been shown to participate in the proteolytic processing of two important neuroendocrine precursors: proopiomelanocortin (POMC) and proinsulin. The gene encoding PC2 has now been cloned and sequenced, and aspects of its expression are being examined. Studies on the evolution of insulin, insulin-related growth factors (IGFs) and their receptors, and the converting enzymes PC2, PC3, and furin are under way in both primitive vertebrates and invertebrates. Studies also are in progress on transgenic mice expressing genes encoding human islet amyloid polypeptide (IAPP), a second product of the β cell that gives rise to islet amyloid deposits in type II diabetics. The structural requirements of the insulin receptor precursor-processing site have been examined and the effect on ligand binding of cleavage between the α and β subunits has been proved to depend on the receptor isoform.

Subtilisin-related Prohormone-converting Enzymes

Intracellular processing of neuroendocrine and peptide hormone precursors usually occurs via cleavage at certain pairs of basic residues, i.e., Lys-Arg or Arg-Arg, by an endoprotease that cleaves on the carboxyl-terminal side of the pair. Carboxypeptidase E or H, an exopeptidase, then removes the basic residues from the newly created carboxyl termini. In neuroendocrine cells such processing occurs mainly in newly formed secretory vesicles of the regulated pathway. Two mammalian prohormone-processing endoprotease(s), PC2 and PC3,

have recently been identified in Dr. Steiner's laboratory. These are serine proteases having a catalytic core that is homologous to that of the Kex2 protease of yeast, which cleaves both the α-mating and killer factor precursors at basic residue pairs.

The PC2 and PC3 proteases were identified by the polymerase chain reaction by using primers based on conserved sequences surrounding essential catalytic residues of Kex2 and the related bacterial subtilisins. Their tissue distribution indicated that both proteases are expressed selectively in neural and neuroendocrine tissues. The full-length cDNAs for PC2 and PC3 were then expressed in *Xenopus* oocytes to define the nature of the encoded proteolytic activity. Both proteases have acidic pH optima and depend on calcium ions for activity. Vaccinia virus vectors have been used to study the activity of PC2 and PC3 on several neuroendocrine precursors, in collaboration with Gary Thomas and his co-workers (Vollum Institute, Portland). These studies indicate that PC2 and PC3 differ significantly in their proteolytic site selectivity; PC3 reproduces the pattern of cleavage of POMC characteristic of the anterior pituitary, while PC2 and PC3 together give a pattern typical of the intermediate lobe. These results are consistent with the patterns of expression of PC3 and PC2 within the pituitary gland. Similar studies in progress on proinsulin indicate that PC2 and PC3 each preferentially cleave at only one of the two basic residue pairs that must be processed in the conversion of proinsulin to insulin.

The gene for human PC2 has been identified and characterized. It is a large gene (>130 kbp in length) and contains 12 exons. The promoter has been partially characterized, and efforts are under way to determine how it is regulated and whether defects in the PC2 gene may occur in some forms of diabetes.

Evolution of Insulin and Insulin Receptor Molecules

The coevolution of insulin, IGFs, and their receptor molecules is currently being explored in both vertebrate and nonvertebrate species. Complementary DNAs encoding prepro-IGF from the hagfish and lamprey (jawless vertebrates) have been identified and sequenced. The IGF molecules from these primitive vertebrates are intermediate in structure between those of human IGF-I and -II, suggesting that they predate the duplication and divergence of the IGF gene. However, a number of features have been conserved, including the presence in lamprey serum of IGF-binding proteins. In amphioxus, a simple ancestral chordate, a preproinsulin-like molecule having features of both vertebrate insulins and IGFs has been identified. This insulin/IGF hybrid molecule may represent an intermediate stage in the divergence of the IGFs from an ancestral "insulin" gene encoding a more typical preproinsulin-like protein. Dr. Steiner and his colleagues are also characterizing the cognate receptors for the above-mentioned hormones by cDNA cloning and *in vitro* expression. Comparison of insulin and IGF receptors in primitive vertebrates should help to identify important structural features that are conserved for specific ligand binding and transmembrane signaling.

Evolution of prohormone convertases. The recent identification of several mammalian proprotein convertases has led to interest in their possible origin and diversification from an ancestral subtilisin-related protease similar to the yeast enzyme Kex2. These enzymes include furin, a widely expressed Golgi-associated protease that appears to process a variety of growth and blood-clotting factor precursors, PC2 and PC3/PC1, prohormone-processing enzymes expressed only in neural and endocrine tissues, as well as several other new members of this subtilisin-related superfamily. Kex2 and furin are both Golgi associated via transmembrane domains and thus seem likely to fulfill a variety of secretory pathway processing functions. The more-specialized PC2 and PC3 enzymes that lack membrane anchors and function only in specialized secretory granules in neuroendocrine tissues appear likely to be later innovations. Dr. Steiner and his colleagues have identified several PC-like enzymes in amphioxus; the initial findings indicate these enzymes have been highly conserved in vertebrate evolution. They have also identified a PC3-like enzyme in the simple metazoan *Hydra vulgaris*, a coelenterate. This finding is of special interest inasmuch as hydra is the simplest organism known to have specialized neural cells secreting neuropeptides related to some of those in more complex eukaryotes.

Proteolytic Maturation of the Insulin Receptor Precursor

Recent studies from Dr. Steiner's laboratory have focused on the proteolytic cleavage of the α/β-proreceptor molecule. A mutagenesis study indicated that only the arginine residues 1 and 4 on either side of the tetrabasic cleavage site $(R_4K_3R_2R_1\downarrow)$ are important for its recognition by the precursor-processing protease, while the internal residues (numbers 2 and 3) are less critical. These results are consistent with the possibility that the proreceptor is processed by a Golgi-associated protease such as furin. The presence of exon 11 allows the expressed proreceptor (mutated at the cleavage site) to be almost fully functional in its binding and tyrosine kinase activity, while proreceptor molecules lacking the 12–amino acid sequence encoded by this small exon (expressed in most tissues) have greatly reduced binding affinity and impaired autophosphorylation. (This research work has been supported by grants from the United States Public Health Service.)

IAPP Expression in Normal Islets and Insulinomas

Recent studies have shown that amyloid deposits in the islets of type II diabetics contain a neuropeptide related to calcitonin gene-related peptide (CGRP). This 37–amino acid peptide (also known as amylin) is expressed in the β cells and is stored in the secretory granules with insulin. Structural variations in the central region of IAPP appear to correlate with species-dependent tendencies to form amyloid deposits in diabetic individuals. Studies on the biosynthesis and processing of prepro-IAPP and the expression of IAPP in islets and various islet cell tumor lines indicate that although insulin and IAPP are synthesized and released together from normal adult islets and βTC3 tumor cells, the expression of IAPP in tumors appears to be up-regulated relative to insulin expression. Transgenic mice expressing human IAPP in the β cells are being studied as a possible model for the development of islet amyloid deposits in type II diabetics. (The work described above is supported by a grant from the United States Public Health Service; the transgenic studies are a collaboration with Dr. Niles Fox at the Eli Lilly Research Laboratories.)

Dr. Steiner is also the A.N. Pritzker Distinguished Service Professor of Biochemistry and Molecular Biology and Medicine at the University of Chicago.

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MEMBRANE TRANSPORT PROCESSES

Michael J. Welsh, M.D., *Investigator*

Cystic fibrosis (CF) is a common lethal genetic disease of Caucasians that is characterized by abnormal electrolyte transport in several organs, including the lung. CF is caused by mutations in the gene that encodes the cystic fibrosis transmembrane conductance regulator (CFTR). Amino acid sequence analysis and comparison with other proteins suggested that CFTR contains five domains: two membrane-spanning domains; one R domain, which contains several consensus phosphorylation sequences; and two nucleotide-binding domains, which are predicted to interact with ATP. Previous work from this and other laboratories indicated that CFTR is a regulated chloride channel.

To understand the biology of CFTR and the pathogenesis of CF, Dr. Welsh's laboratory did several studies focused on the function of CFTR and its individual domains. Previous studies had shown that the CFTR chloride channel is regulated by agents that increase cellular cAMP. In addition, a CFTR mutant in which the R domain was deleted produced a channel that was open, even without an increase in cellular levels of cAMP. To understand further how the R domain regulates CFTR, the laboratory studied excised, inside-out patches of membrane from cells expressing recombinant CFTR. Addition of the catalytic subunit of cAMP-dependent protein kinase (PKA) and ATP to the cytosolic surface activated CFTR chloride channels. This indicated that the channel is regulated by phosphorylation.

To identify the R domain as the site of phosphorylation and to identify specific residues responsible for channel activation, each of the 10 potential PKA phosphorylation sites was mutated individually. The consensus sequence for PKA-dependent phosphorylation is Arg/Lys-Arg/Lys-X-Ser/Thr; at each potential site, Ser/Thr was mutated to Ala. The studies showed that upon stimulation with cAMP, four serines were phosphorylated *in vivo*; all are located in the R domain. Mutation of any single serine did

not prevent cAMP from opening the channel. However, when all four serines were mutated, the channel failed to respond to cAMP agonists. Nevertheless a triple mutant containing only one of the four serines still responded to cAMP. These results led to the conclusion that phosphorylation-dependent regulation of the CFTR chloride channel is degenerate. That is, normally more than one site is involved, but no single site is essential. Furthermore a single site alone may be sufficient. This is a novel mechanism of regulation not described for any other channel.

In CFTR, the function of the nucleotide-binding domains (NBDs) has been an enigma. On the one hand, the NBDs are the conserved feature of the CFTR amino acid sequence that suggests that it belongs to a family of proteins, many of which serve as pumps. On the other hand, why should an ion channel contain a domain that might hydrolyze ATP? Adding to the mystery, the NBDs are the site of a majority of naturally occurring CF mutations. To discover the function of the NBDs, the Welsh laboratory tested the hypothesis that ATP would regulate the CFTR chloride channel. They found that once the channel was phosphorylated by cAMP-dependent protein kinase, ATP is required to keep it in the open state. Moreover, because only hydrolyzable analogues of ATP were effective, the data suggested that ATP hydrolysis may be required for the channel to open. That result suggested that some CF-associated mutations in the NBDs may cause dysfunction of CFTR by disrupting its normal regulation.

Some further clues about the function of CFTR and abnormalities in disease came from the studies of its cellular location. Antibodies to CFTR were used to localize CFTR. By using confocal laser scanning microscopy, CFTR was identified in the apical region of several chloride-secreting intestinal epithelial cell lines. More direct evidence that CFTR is located within the apical membrane came from studies showing that an antibody directed against an extracellular epitope labeled the apical membrane of unpermeabilized intestinal epithelial cell lines. Localization of CFTR in the apical membrane places it in a position where it can directly mediate chloride transport; for a chloride channel to mediate chloride transport directly it must be located in the apical membrane.

Numerous mutations are reported to cause CF, but deletion of phenylalanine at position 508 (ΔF508) is the most common. Previous studies from the Welsh and other laboratories suggested that CFTR ΔF508 is not completely processed. Those observations suggested that the mutant proteins are retained in the endoplasmic reticulum rather than being transported to the plasma membrane. As a result the apical membrane would not contain functional chloride channels. To test the hypothesis that such mutants are not delivered to the plasmid membrane, Dr. Welsh and his colleagues used primary cultures of airway epithelia grown on permeable supports; under these conditions they polarized and expressed the CF defect in apical chloride permeability. They developed a semiquantitative assay using nonpermeabilized epithelia, an antibody directed against an extracellular epitope of CFTR, and large fluorescent beads bound to secondary antibodies. They observed specific binding to airway epithelia from non-CF subjects, indicating that CFTR is located in the apical membrane of airway epithelia. In contrast, there was no specific binding to the apical membrane of CF airway epithelia. These data were supported by qualitative studies using confocal microscopy: the most prominent immunostaining was in the apical region of non-CF cells and in the cytoplasmic regions of CF cells. The results indicate that CFTR is either missing from the apical membrane of the CF cells or is present at a much reduced level. Thus those results explain the lack of chloride permeability in most CF airway epithelia.

Dr. Welsh is also Professor of Internal Medicine and of Physiology and Biophysics at the University of Iowa College of Medicine, Iowa City.

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MOLECULAR BIOLOGY OF GROWTH FACTOR SIGNAL TRANSDUCTION

Lewis T. Williams, M.D., Ph.D., *Investigator*

Dr. Williams's group previously developed an approach to mapping sites of interaction between intracellular signaling molecules and receptor tyrosine kinases. Short tyrosine-phosphorylated peptides representing receptor sequences were used to block the binding of signaling molecules to the receptor *in vitro* and thereby identify the receptor sequence that binds each signaling molecule. Using this principle, the group showed that it was possible to inactivate an individual intracellular signaling pathway selectively by mutating the specific receptor tyrosine that binds the signaling molecule that triggers the pathway. This year these and other approaches were used to learn more about receptor-triggered interactions of signaling molecules.

Phosphatidylinositol 3-Kinase

Using a platelet-derived growth factor (PDGF) receptor point mutant (Y708/719F) that was shown previously by Dr. Williams's group to be selectively defective in binding phosphatidylinositol 3-kinase (PI 3-kinase), they recently found that this receptor mutant was unable to stimulate Raf-1 and microtubule-associated protein (MAP) kinases. This mutant

receptor was also defective in activating p21 *ras*. These results provide an explanation for the previous observation that PDGF receptors that have mutated PI 3-kinase–binding sites are unable to stimulate mitogenesis and suggest that PI 3-kinase regulates p21 *ras* activity as well as Raf-1 and MAP kinases.

Although Dr. Williams's group showed previously that PI 3-kinase binds to activated PDGF receptors, it was not known whether the catalytic activity of PI 3-kinase was increased by this interaction. This year, Dr. Jaime Escobedo showed that purified receptor stimulated a 10-fold increase in activity of purified PI 3-kinase *in vitro*. He also showed that the enhancement of PI 3-kinase *in vitro* required tyrosine phosphorylation of the PI 3-kinase by the receptor. Both the 85- and 110-kDa subunits were found to be tyrosine-phosphorylated in PDGF-stimulated cells. The conclusion was that PI 3-kinase is activated by tyrosine phosphorylation.

Role of Phospholipase C-γ with Fibroblast Growth Factor Receptor Signaling

In previously proposed models of signal transduction by fibroblast growth factor (FGF) receptors, phospholipase C-γ (PLC-γ) was thought to play a pivotal role. By testing candidate tyrosine-phosphorylated peptides for their ability to block binding of PLC-γ to the FGF receptor, Dr. Williams's group showed that tyrosine 766 is the binding site for PLC-γ on the FGF receptor. They then mutated this site and found that the mutated receptors lost the ability to stimulate PI hydrolysis and could not elicit an increase in cytosolic calcium. This showed that association of PLC-γ with receptors was essential for growth factor–induced calcium elevations that were previously thought to be essential for mitogenesis. However, the mutated FGF receptor elicited a normal mitogenic response and also activated Raf-1 and MAP kinases normally. It was concluded that neither calcium elevation nor PI turnover was required for the mitogenic effect of FGF or for FGF-stimulated MAP kinase and Raf-1 kinase activities. These conclusions refuted previously proposed models of FGF-stimulated signal transduction.

Raf Kinase Mediates FGF-stimulated Mesoderm Induction

Recent work from Dr. Marc Kirschner's group showed that FGF is important in the induction of mesoderm and in the formation of posterior structures during *Xenopus* embryonic development. Dr. Williams's group investigated the role of Raf-1 ser-ine/threonine kinase in FGF-stimulated mesoderm induction, and Dr. Angus MacNicol constructed mutated Raf-1 protein (Naf) that inhibited the activity of wild-type Raf-1. He expressed Naf in *Xenopus* embryos by injecting Naf mRNA into both cells of the two-cell-stage embryo. At stage 8 of development, explants of the animal hemisphere were exposed to FGF or activin and examined for the induction of mesoderm *in vitro*. The mesoderm-inducing effect of FGF was blocked in embryos that expressed Naf. However, the induction of mesoderm by activin, a factor that acts by different signaling pathways, was not affected by Naf. The developmental consequences of allowing Naf-injected embryos to progress through later stages of embryogenesis were then examined. At the tadpole stage the Naf-injected embryos had normal eyes and cement glands (head structures), as well as normal hearts, but had severe truncations of their tails. This phenotype is remarkably similar to the previously reported phenotype of embryos that had their FGF receptors blocked. This is the first demonstration of a role for Raf-1 in vertebrate development and in a signaling pathway stimulated by FGF.

Dr. Anthony Muslin tested the role of Raf-1 in another process, the maturation of *Xenopus* oocytes induced by progesterone. He found that progesterone stimulated a fourfold increase in Raf-1 kinase activity. Injection of the RNA encoding an oncogenic raf (v-*raf*) caused oocytes to mature even in the absence of progesterone. When dominant negative raf (Naf) RNA was injected, oocyte maturation induced by progesterone was blocked. The activation of MAP kinase was also blocked. These findings suggest that Raf-1 plays an important role in oocyte maturation and in the activation of MAP kinase.

Cloning of the cDNA for a Receptor for Vascular Endothelial Growth Factor

Vascular endothelial growth factor (VEGF) acts exclusively on endothelial cells to stimulate their proliferation and to increase endothelial permeability. Dr. Williams's group cloned a new cDNA that encodes the VEGF receptor and showed that it defined a new class of receptor tyrosine kinase. The mRNA for this receptor was expressed only in endothelial cells and was especially prominent in the primitive endothelial cells that line blood islands in the mouse yolk sac and in new blood vessels formed in healing wounds. This unique pattern of expression suggests a role of VEGF receptor in angiogenesis. (This work was supported by a grant from the National Heart, Lung and Blood Institute, National Institutes of Health.)

Inhibition of FGF Receptor Function in Skin of Transgenic Animals

Dr. Williams's group used skin as a model system for inhibiting FGF function in a transgenic mouse by targeted expression of a dominant negative mutant FGF receptor that inhibits the function of wild-type FGF receptors. Using the keratin 10 promoter, they achieved localized expression of the mutant receptor in the suprabasal layer of the skin and thereby specifically blocked the action of FGF in this region. The suprabasal cells of these animals did not differentiate properly and proliferated excessively. Histologically the skin resembled that of patients with hyperproliferative skin diseases. These studies, which showed that FGF plays a role in promoting skin differentiation, were supported by a grant from the National Heart, Lung and Blood Institute, National Institutes of Health.

Alternatively Spliced Forms of FGF Receptor Have Different Ligand Specificities

Dr. Williams's group previously showed that the FGF receptor genes encode multiple receptors that differ in their extracellular domain sequences and are generated by alternative splicing. This year they found that each splice variant has a distinctive specificity pattern in binding selected members of the FGF family of factors. They also showed that it is possible to inactivate selectively the response of an endogenous FGF receptor to a specific ligand in the FGF family by coexpressing a dominant negative mutant receptor that selectively binds that ligand.

Dr. Williams is also Professor of Medicine at the University of California, San Francisco.

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PATHOGENESIS AND TREATMENT OF INHERITED DISEASES

James M. Wilson, M.D., Ph.D., *Assistant Investigator*

The overall goal of Dr. Wilson's research is to de-
fine the pathogenesis of human inherited diseases
and to develop therapies based on somatic gene
transfer. In the past year he has made progress in two
model systems.

Familial Hypercholesterolemia

An important area of research in Dr. Wilson's labo-
ratory has been the development of gene replace-
ment therapies based on gene transfer into the liver.
An inherited deficiency of the receptor for low-
density lipoproteins (LDLs), which leads to the fa-
milial hypercholesterolemia (FH) syndrome, has
been used as a model for such development. Critical
to this effort has been the availability of an animal
model for FH, the Watanabe heritable hyperlipid-
emic (WHHL) rabbit. The initial strategy for genetic
treatment of FH is based on transplantation of autolo-
gous hepatocytes that have been genetically
corrected *ex vivo* with recombinant retroviruses.

The feasibility of *ex vivo* gene therapy of FH was
established in experiments with WHHL rabbits. He-
patocytes harvested from these animals were plated
in primary cultures and exposed to recombinant ret-
roviruses capable of transferring a functional rabbit
LDL receptor gene efficiently. These genetically
modified cells were then injected into the portal
vein of the animal of origin. The transplant recipi-
ents demonstrated substantial levels of recombinant
LDL receptor RNA in their liver for the duration of
the experiment, which was terminated six months
after initial cell transplantation. Transplant recipi-
ents realized a 20–50% diminution in serum choles-
terol, which persisted for at least four months.

The results obtained with the WHHL model en-
couraged Dr. Wilson and his co-workers to under-
take a series of experiments in preparation for a
clinical trial of gene therapy against homozygous
FH. Techniques for isolating human hepatocytes

were established, and recombinant retroviruses ca-
pable of transducing a fully functional LDL receptor
gene were produced. Pilot experiments were per-
formed in baboons to assess the safety and toxicity of
ex vivo, liver-directed gene therapy. A clinical pro-
tocol for treating patients with severe homozygous
FH, based on transplantation of autologous, geneti-
cally corrected hepatocytes proposed by Dr. Wilson
and his colleagues was approved by the Recombi-
nant DNA Advisory Committee of the National Insti-
tutes of Health and the Food and Drug Administra-
tion. The first patient was treated in June 1992.
Grants from the National Institutes of Health pro-
vided support for the program in FH gene therapy.

An alternative approach to liver-directed gene
therapy of FH that may be more effective and less
morbid is the direct delivery of a functional LDL
receptor gene to hepatocytes *in vivo*. One promis-
ing approach, which Dr. Wilson and his colleagues
are pursuing, is the development of gene transfer
substrates composed of DNA-protein complexes. A
ligand for the liver-specific asialoglycoprotein re-
ceptor is conjugated to DNA of an expression vector.
Initial experiments analyzed the expression of a re-
porter gene in tissues of rats that had been injected
intravenously with a DNA-protein complex.

Strategies for obtaining stable expression of the
transgene *in vivo* have been identified. This is ac-
complished by subjecting the animal to partial hepa-
tectomy immediately after injection of the complex.
Analysis of DNA from liver tissue of animals that
stably express the recombinant gene revealed some
unexpected results. The recombinant gene abun-
dantly persists in liver tissue for at least four months
as episomal DNA that retains the structural and meth-
ylation pattern of the input plasmid.

Studies are under way to use this approach to
correct the genetic defect in several animal models
of human diseases, including LDL receptor gene

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transfer in the WHHL rabbit, factor IX gene transfer in a canine model of hemophilia B, and ornithine transcarbamylase gene transfer in a mouse model of hyperammonemia. The use of this technology to treat ornithine transcarbamylase deficiency has been supported by the National Institutes of Health.

Cystic Fibrosis

The recent isolation of the gene responsible for cystic fibrosis (CF) has provided exciting opportunities to study the pathogenesis of the disease and to design new strategies for therapy based on somatic gene transfer.

CF is an autosomal recessive disease marked by defective salt and water transport across epithelia of a variety of organs. The protein product of the CF gene, the cystic fibrosis transmembrane conductance regulator (CFTR), functions in part as a chloride channel in the plasma membrane.

Soon after the isolation of the CF gene by other investigators, Dr. Wilson began a series of projects whose long-range goal is to treat the pulmonary manifestations of CF by gene therapy directed to the airway epithelial cells. The first step was to reconstruct a normal CF gene and clone it into a recombinant retrovirus capable of efficiently transducing human cells. Pancreatic cells isolated from a CF patient regained normal physiologic functions (i.e., cAMP-regulated transport of chloride) when exposed to the CFTR-transducing retrovirus.

In order to design rational strategies for reconstituting CFTR expression in the lungs of CF patients, it was necessary to define precisely the distribution of endogenous CFTR expression in the human lung. Techniques of *in situ* hybridization and immunocytochemistry were used to localize CFTR in human lung specimens. Analysis of the proximal airway revealed low levels of CFTR expression in the surface epithelium, with substantially higher expression in cells of submucosal glands, structures that produce large quantities of mucus. High levels of CFTR were also detected in a subpopulation of surface epithelial cells that populate bronchioles of the distal airway as well as a minority of the cells that line alveoli.

Critical to the study of CF lung pathogenesis and the development and characterization of approaches to gene therapy is the availability of an authentic animal model of the human airway. Epithelial cells derived from human non-CF and CF airways are harvested from lung tissue and seeded into denuded rat trachea, which are then implanted into athymic (*nu/nu*) mice. Xenografts develop fully differentiated human epithelium within 3–4 weeks. This model has been used to evaluate the feasibility of gene therapy with several recombinant viruses. Efficient and stable genetic reconstitution was only obtained with recombinant retroviruses when they were delivered to an undifferentiated, regenerating xenograft; essentially no gene transfer was detected when the xenograft epithelium was fully differentiated at the time of gene transfer. In contrast, recombinant adenoviruses were capable of efficiently transferring a CFTR transgene into cells of a fully differentiated epithelium. Furthermore, transgene expression was stable for the lifetime of the grafts, which was usually 4–6 weeks.

Dr. Wilson is also Associate Professor of Internal Medicine and Biological Chemistry at the University of Michigan Medical School.

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POST-TRANSCRIPTIONAL CONTROL OF GENE EXPRESSION

Sandra L. Wolin, M.D., Ph.D., *Assistant Investigator*

Dr. Wolin's laboratory is interested in understanding post-transcriptional mechanisms for regulating eukaryotic gene expression. In one project, Dr. Wolin and her colleagues are studying mechanisms that regulate the translation of mRNAs into proteins. In a second project, they are investigating the structure and function of a conserved class of small cytoplasmic ribonucleoprotein particles.

Translation Mechanisms

It has long been observed that the movement of ribosomes along mRNA during translation is not linear with time. Rather, ribosomes pause at discrete sites for unknown reasons. Several possible causes of ribosome stalling are mRNA sequence and structure as well as activities of trans-acting factors in the translation reaction. The importance of these factors in regulating the movement of ribosomes along the mRNA has been relatively unexplored, perhaps because of the absence of an assay sensitive enough to detect subtle changes in ribosome movement. The laboratory has developed and is currently using such an assay to probe the dynamics of ribosome movement during translation.

A newly revealed slow point in translation initiation. The laboratory has shown that one major position of ribosome pausing, in both reticulocyte and wheat germ extracts, is directly over the initiation codon of the mRNA. This pausing by fully assembled ribosomes appears to represent a slow step in eukaryotic protein initiation that has not been previously detected. This slow point in translation is a bona fide intermediate in translation, because >50% of the ribosomes that pause at this position continue to move down the mRNA. By using initiation inhibitors that are specific for individual steps in translation, it has been determined that the first peptide bond has been synthesized but has not yet been translocated from the A site to the P site of the ribosome. Thus the first elongation factor 2 (EF-2)-dependent translocation event appears to be a rate-limiting step in the translation of a variety of different mRNAs *in vitro*. Recent studies suggest that this slow point in translation also occurs *in vivo*.

What features of mRNA sequence result in ribosome pausing and frameshifting? Because codon usage is strongly correlated with the relative abundance of the respective tRNA species, it has frequently been suggested that ribosome pausing occurs at rare codons. To study this question, the laboratory has prepared translation extracts in which tRNA molecules and amino acids have been removed. By adding back uncharged tRNA and dif-

ferent populations of amino acids, the levels of individual charged tRNAs in the translation extract can now be manipulated. These studies should lead to an elucidation of the role that rare tRNAs play in ribosome pausing during translation.

A tertiary structure in mRNA, known as an RNA pseudoknot, has also been postulated to force ribosomes to pause during translation. This RNA structure is required for ribosome frameshifting during translation of certain retroviral mRNAs. The pseudoknot is thought to cause ribosomes to pause over certain "slippery" sequences in the mRNA, resulting in frameshifting. Two retroviral mRNAs are currently being examined to determine if ribosomes actually pause at the site of the frameshift and if this pausing is due to the presence of a pseudoknot.

The Ro Small Ribonucleoprotein Particles

All eukaryotic cells contain an array of small RNA-protein complexes that play fundamental roles in cell metabolism. These ribonucleoprotein particles (RNPs) are often classified based on their subcellular location, i.e., small nuclear ribonucleoproteins (snRNPs), small cytoplasmic ribonucleoproteins (scRNPs), and small nucleolar ribonucleoproteins (snoRNPs). Some of the best-characterized small RNPs are the small nuclear U RNPs, which are involved in mRNA processing, and the cytoplasmic signal recognition particle, which is important for targeting nascent secretory proteins to the endoplasmic reticulum membrane.

In addition to these relatively well-characterized small RNPs, there are additional small RNPs in cells whose functions remain obscure. One class of these RNPs, the Ro RNPs, was discovered because these particles are frequent targets of the autoimmune response in patients with two rheumatologic disorders, systemic lupus erythematosus and Sjögren's syndrome. The Ro RNPs (named because they are immunoprecipitated by anti-Ro lupus antibodies) consist of several small RNA molecules of ~100 nucleotides in length, each of which is complexed with a 60-kDa polypeptide. Each Ro RNP is present in ~10^5 copies per cell, or ~1% the number of ribosomes.

To define possible functions of the Ro RNPs, it is important to know their distribution within cells. Immunofluorescence experiments with patient autoantibodies have long given conflicting results, presumably because of the multiple specificities present in patient sera. Thus, to determine definitively the subcellular location of Ro RNPs, the distribution of these particles was determined in enucleated mammalian cells and karyoplasts. In these cell enucleation experiments, the Ro RNPs appear to be primarily cytoplasmic. As a further test, nucleic acid probes specific for the RNA components of these particles are being used to determine the distribution of Ro RNPs within the cytoplasm, in relation to known organelles. This information will be valuable in making predictions for Ro RNP function.

The Ro RNPs must function in a basic cellular process, because they have been found in every vertebrate cell type examined. Cloning and sequencing studies of the *Xenopus* protein and RNA components have revealed that the human and *Xenopus* components are nearly 80% identical, indicating that these particles are extremely conserved cellular components. *Xenopus* eggs have been demonstrated to contain stores of the 60-kDa Ro protein, which can be used to assemble heterologous Ro RNPs by mixing egg extracts with RNAs from other species. Future work will focus on identifying molecules that interact with Ro RNPs and on identifying Ro RNPs in species amenable to genetic analysis.

Dr. Wolin is also Assistant Professor of Cell Biology at the Yale University School of Medicine.

ENDOTHELINS AND THEIR RECEPTORS

Masashi Yanagisawa, M.D., Ph.D., *Associate Investigator*

The endothelins are a family of three small peptides with a variety of potent biological activities that are mediated by their specific G protein–coupled receptors. Dr. Yanagisawa and his colleagues earlier identified the original member of the family, endothelin-1, a strong and extremely long-lasting vasopressor molecule secreted by vascular endothelial cells. This year the laboratory has initiated a number of projects aimed at further characterization of the physiological role and regulation of the endothelins and their receptors.

The Endothelin/Endothelin-Receptor System

Despite the relative wealth of information regarding molecular components of the endothelin/endothelin-receptor system, its regulation, and its

pharmacological activities at the cellular and isolated-tissue levels, little is known about its physiological significance as a regulatory system in living animals. Although evidence suggests that the endothelins may play important roles in certain pathologies involving abnormal vascular reactivity, it is unknown whether the peptides actually participate in homeostatic regulation (of, for example, blood pressure) under healthy conditions. Because endothelins and their receptors are expressed in a number of nonvascular cell types, including endocrine cells and neurons, it is possible that blood vessels may represent only one of the many stages in which the peptides can play a crucial regulatory role. Although a few endothelin receptor antagonists have become available recently, they have not proved useful in studying the local interaction of endogenously produced endothelins and receptors. To approach these questions, Dr. Yanagisawa's laboratory has started a project to knock out endothelin and endothelin receptor genes by homologous recombination in the mouse. The laboratory has cloned the mouse genes for the two endothelin receptor subtypes, called ET_A and ET_B, constructed the targeting vectors, and is now producing homologous-recombinant embryonic stem cells. The work will be conducted as a collaboration with Dr. Robert E. Hammer (HHMI, University of Texas Southwestern Medical Center at Dallas) and Dr. Joachim Herz (also at Dallas), whose team has already produced several mouse strains harboring other targeted genes.

Molecular Identification
of Endothelin-converting Enzyme

Like many other peptide hormones and neuropeptides, endothelins are processed from the corresponding precursor (prepro-) proteins. However, biologically active 21–amino acid endothelins are produced via a formerly unknown type of proteolytic processing: approximately 40-residue, biologically inactive intermediates, called big endothelins, are first excised from the prepro-endothelins. The carboxyl-terminal halves of the big endothelins are then cleaved off between Trp21 and Val/Ile22 to produce the amino-terminal active peptides. This unusual endoproteolytic activation is catalyzed by endothelin-converting enzyme(s), a membrane-bound metalloprotease(s) that is yet to be identified. The activity of this enzyme requires neutral pH and is sensitive to EDTA and the metalloprotease inhibitor phosphoramidon but is apparently distinct from any other proteases known, including the enkephalinase or neutral endopeptidase 24.11. Because big endothelin-1 is virtually inactive if the

conversion is inhibited, the enzyme could also be an important target for the possible pharmacological intervention to the system. Answers to many questions about the biosynthetic pathway and regulation of the endothelins await the identification of this protease.

Dr. Yanagisawa's laboratory has aimed at the molecular characterization of the converting enzyme by employing various approaches. To purify the enzyme at the protein level, the laboratory has established a rapid assay for the enzyme activity and conducted a systematic search for a suitable starting material. The laboratory has also established cell lines that produce a large amount of big endothelin-1 without secreting a detectable level of the mature peptide. These cell lines should be suitable as host cells for the transfection of expression cDNA libraries from endothelial cells and other tissues rich in the converting enzyme.

Dr. Yanagisawa is also Associate Professor of Molecular Genetics at the University of Texas Southwestern Medical Center at Dallas.

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GENETICS

The Institute's Program in Genetics remains the largest of its five program areas, and it intersects extensively with each of the other four. Work carried out ranges from basic studies at the molecular and cellular levels, through developmental pathways, to clinically based studies of human genetic disorders and potential new therapeutic approaches. Investigators within this program are located at Emory University, the University of Michigan, Johns Hopkins University, Indiana University at Bloomington and at Indianapolis, Harvard Medical School, Children's Hospital at Boston, the University of Colorado at Boulder, the Massachusetts Institute of Technology, the University of Chicago, Duke University, Baylor College of Medicine, the University of Iowa College of Medicine at Iowa City, Yale University, Rockefeller University, Stanford University, the California Institute of Technology, the University of Pennsylvania, Princeton University, the University of Utah, the University of Washington in Seattle, Fred Hutchinson Cancer Research Center in Seattle, Washington University in St. Louis, Brandeis University in Waltham, and the University of California at Berkeley and at San Francisco.

The molecular basis of human genetic disease is the theme of the research of Investigator Francis S. Collins, M.D., Ph.D. (University of Michigan) and his colleagues. Use of the positional cloning approach has made it possible to identify genes responsible for inherited disease, even when there is little or no information available about the basic biological defect. This strategy was successful in yielding the genes for cystic fibrosis and neurofibromatosis over the past three years, and details of the basic biological defects in those two diseases are now becoming much clearer. The positional cloning approach is being intensively utilized to search for genes for familial breast cancer on chromosome number 17 and for Huntington's disease on chromosome 4. In both of these searches the relevant interval has been narrowed to a manageable stretch of genomic DNA, and the identification of the responsible genes is close at hand.

The adenomatous polyp represents an early stage in the development of colon cancer in the general population as well as in families where inheritance of a mutation causing numerous polyps leads to a high incidence of colon carcinoma. The recently cloned gene (APC) responsible for the inherited condition is therefore of broad interest, and ongoing experiments by Investigator Raymond L. White, Ph.D. (University of Utah) and his colleagues are

yielding information about its normal function and the consequences of various mutations. The techniques of genetic and physical mapping that led to the successful isolation of APC, and the experience gained in that endeavor, will speed the eventual identification of other genes that play roles in human genetic disease.

The laboratory of Assistant Investigator David C. Page, M.D. (Massachusetts Institute of Technology) has investigated the structure and function of the mammalian genome and its role in embryonic development. Efforts were focused largely on the human and mouse X and Y chromosomes and were directed at understanding genes involved in Turner syndrome and in sex determination. Comprehensive genetic maps of the human Y chromosome were generated; the methods developed should be applicable to the mapping of all human chromosomes. A pair of genes located on the human X and Y chromosomes was implicated in Turner syndrome, one of the most common chromosomal disorders. Mutations were identified that cause XX embryos to develop as males or cause XY embryos to develop as females.

Investigation of genetic diseases of the human X chromosome is the major interest of the laboratory of Associate Investigator Stephen T. Warren, Ph.D. (Emory University). Work continues to unravel the unusual mutation responsible for the fragile X syndrome, the most frequently encountered form of human mental retardation. This novel mutation is the extraordinary expansion in patients of a 3–base pair repeat within the FMR-1 gene from a normal average of 29 repeats to beyond 1,000 repeats. Carriers display intermediate levels of expansion that are remarkably unstable when transmitted from parent to child. The actual length of the repeat in carriers influences the chance of having an affected child and resolves the unusual genetics of fragile X syndrome, termed the Sherman paradox. Progress has also been made in the investigation of Emery-Dreifuss muscular dystrophy (EDMD). An excellent candidate gene has been found that maps quite close to the EDMD mutation and encodes a protein similar to dystrophin, a protein involved in other X-linked muscular dystrophies.

Investigator C. Thomas Caskey, M.D. (Baylor College of Medicine) and his colleagues are studying the mechanisms of genetic disease and seek to improve procedures for diagnosis and therapy. Their research over the past year has identified the gene defect for myotonic dystrophy and revealed the mechanism for mutation in humans, termed triplet

repeat expansion, which has been found to operate in the major mental retardation disease of fragile X syndrome and in myotonic dystrophy. This points the way technically toward the isolation of other disease genes by simple methods. Dr. Caskey's laboratory will now expand its search to cancer and the genes of aging. Mouse genetic technology now allows testing of human disease mechanisms and treatment with speed and precision.

Investigator Louis M. Kunkel, Ph.D. (Children's Hospital, Boston) and his associates are continuing their study of dystrophin and dystrophin-related proteins. Dystrophin is the protein disrupted by mutation that gives rise to Duchenne/Becker muscular dystrophy. The related proteins are likely to be involved in the generation of other neuromuscular genetic diseases. They are also proteins with the potential of replacing dystrophin's function in diseased muscle and represent a potential avenue for therapeutic intervention. The overall aims of the laboratory are to identify new neuromuscular disease genes and to design means of treating children affected with muscular dystrophy.

The research in the laboratory of Associate Investigator Robert L. Nussbaum, M.D. (University of Pennsylvania) has been directed in the past toward elucidating the molecular bases for three human genetic diseases that cause mental retardation and/or abnormalities in vision or hearing: fragile X syndrome, choroideremia, and the Lowe oculocerebrorenal syndrome. Each disease under investigation was known to be caused by a gene on the X chromosome, but the molecular mechanism, the gene involved, and the nature of the underlying mutations have been hitherto unknown. Work by this group and in other laboratories has allowed the identification and isolation of the genes responsible for all three of these diseases. The laboratory will now concentrate on studying the gene responsible for the Lowe oculocerebrorenal syndrome and the normal processes that are disrupted by defects in this gene. The group is also undertaking an analysis of a human gene that shows strong similarity to certain genes in yeast and fruit flies that encode a class of transcription factors known as global activators of transcription.

Investigator David L. Valle, M.D. (Johns Hopkins University) and his colleagues have continued their studies of genes involved in human genetic disease. In particular, they have examined the cell biology, expression, and genetic defects of the ornithine-δ-aminotransferase gene. Deficiency of this enzyme disrupts ornithine metabolism and causes an inherited, blinding, progressive chorioretinal degeneration known as gyrate atrophy of the choroid and

retina. Other genes important for retinal function, including the human homologue of recoverin, have been cloned, and their possible role in retinal degeneration is being explored. Finally, molecular studies of genes involved in peroxisomal biogenesis are ongoing. The genes for two peroxisomal integral membrane proteins have ben cloned. One, which encodes the 70-kDa peroxisomal membrane protein, appears to be responsible for the inborn errors of peroxisomal biogenesis in a subset of patients.

Associate Investigator Jeremy Nathans, M.D., Ph.D. (Johns Hopkins University) and his colleagues are investigating the function and development of the human retina, the light-absorbing tissue in the eye that carries out the first steps in vision. This past year his laboratory succeeded in producing the light-sensing compounds, called visual pigments, that mediate color vision. By producing and characterizing the visual pigments found in those 6–8% of males with anomalous color vision, Dr. Nathans was able to define precisely the way in which their vision differs from that of the majority of the population. In other work, the laboratory has continued its analysis of the genetic defects responsible for retinitis pigmentosa, a progressive degeneration of the retina that affects 1 person in 4,000. Thus far, 18 different mutations have been identified in the gene encoding rhodopsin, the visual pigment mediating dim light vision. Biochemical studies of these mutant proteins show that there are at least two different types of defect.

Hearing loss is the most common form of sensory impairment. Profound childhood hearing loss in human populations has an incidence of 4–8/1,000 births in developed countries, with the likely etiology of a single gene mutation in at least half. An estimated 5% of school-age children have unilateral and/or mild-to-moderate hearing loss, representing a potentially reversible cause of learning difficulty. Progressive hearing loss, or presbycusis, occurs as part of the normal aging process, with a one in six chance of functionally significant hearing loss by age 65. While environmental causes such as acoustic trauma, infection, and ototoxic drugs play a significant causative role in auditory sensory impairment, underlying predisposing genetic factors are likely. Some types of genetic hearing loss can easily be distinguished, since they occur in association with other features as part of a syndrome and are a major component in more than 100 defined genetic disorders. Nonsyndromic or undifferentiated hearing loss represents the second major category of hearing loss in which auditory sensory impairment is an isolated finding. Analysis of such families is complicated by the difficulty in determining

whether the hearing loss in affected family members is genetic or acquired. If the hearing loss is proved to be hereditary, analysis of pedigrees may be further complicated by genetic heterogeneity and nonassortive mating. The broad goal of the laboratory of Assistant Investigator Geoffrey M. Duyk, M.D., Ph.D. (Harvard Medical School) is to develop the methods and resources to identify and study the genetic basis for nonsyndromic hearing loss. Toward these ends, the research focuses on the construction of a very high resolution human genetic linkage map and definition of the basis for hearing at the molecular level.

The mapping of genes of known function to sites on human and mouse chromosomes can lead to identification of candidate genes for human genetic disorders or inherited mouse mutations. The gene for peripheral myelin protein (PMP-22) has been mapped to mouse chromosome 11 and human chromosome 71p by the laboratory of Investigator Uta Francke, M.D. (Stanford University) and was subsequently shown to be mutated in the dominant neurologic mutation *Trembler* in the mouse. The gene is also likely to contribute to the hereditary motor and sensory neuropathy Charcot-Marie-Tooth disease, type 1A, since it is located on the small duplicated DNA segment that is associated with this disease. A candidate gene for contributing to the Prader-Willi syndrome phenotype was identified by mapping the small nuclear ribonucleoprotein polypeptide N gene to the submicroscopic deletion region critical for this syndrome on chromosome 15q. The gene is imprinted on the maternally derived chromosome in mice. Molecular genetic analysis of a human X-autosome translocation identified an associated deletion and conserved sequence motifs at all three breakpoints. This is the second human constitutional translocation that has been analyzed at this level, and the results suggest possible mechanisms. In an induced translocation in transgenic mice, clusters of transgenes were localized to chromosomal sites on both translocation chromosomes, again suggesting models regarding the origin of this translocation event. In the growth hormone insensitivity syndrome, a rare autosomal recessive disorder, additional mutations in the growth hormone receptor gene were identified in patients from different parts of the world. These data throw light on the origin and nature of mutations that lead to a severe growth deficiency.

Common cardiovascular disorders such as coronary artery disease and hypertension constitute broad clinical classes representing multiple etiological entities, where many genes and environmental determinants are likely to be involved. Work on the genetics of hypertension in the laboratory of Investi-

gator Jean-Marc Lalouel, M.D., D.Sc. (University of Utah) follows two directions: molecular investigations of rare Mendelian syndromes of hypertension and linkage analysis in a large series of hypertensive siblings. In glucocorticoid-remediable aldosteronism, a rare disorder marked by severe hypertension and high levels of abnormal steroid hormones, an unequal crossing over between genes involved in the biosynthesis of adrenal steroids creates a new chimeric gene with expression that explains the observed pathophysiology. Linkage studies of essential hypertension are under way and have first concentrated on the renin-angiotensin system.

Investigator Arthur L. Beaudet, M.D. (Baylor College of Medicine) and his colleagues use recombinant DNA techniques to study human genetic diseases. One project concerns the role of proteins that mediate adhesion of white blood cells to blood vessel walls. Mutations are being prepared in mice to study the genes encoding these proteins in models of various inflammatory diseases and atherosclerosis. Naturally occurring genetic variation in the human population is being evaluated to examine the role of these genes in common adult disease processes. In other studies, automated methods for DNA testing are being developed in order to screen for couples at risk for having a child affected with cystic fibrosis. A mutant mouse model for cystic fibrosis also is under development. Other projects are under way to clone the genes for two human conditions associated with mental retardation, the Prader-Willi and Angelman syndromes.

Investigator Graeme I. Bell, Ph.D. (University of Chicago) and his colleagues are studying the causes of non-insulin-dependent, or type 2, diabetes mellitus (NIDDM). Genetics and molecular biology are being used to identify and characterize the genes that increase the risk of developing this common disorder of aging adults. Genetic studies of families with a highly penetrant, autosomal dominant form of NIDDM that may affect children and adolescents, termed maturity-onset diabetes of the young (MODY), have identified diabetes-susceptibility genes on chromosomes 7 and 20. The locus on chromosome 7 encodes the glycolytic enzyme glucokinase, which plays an important role in regulating and integrating glucose metabolism in insulin-secreting cells of the pancreas and in the liver. Twenty different mutations in the glucokinase gene associated with the development of NIDDM have been identified to date in French, British, Swedish, Japanese, and African-American patients, indicating that they are not restricted to a particular ethnic or racial group. The identity of the gene on chromosome 20 has not been determined, and studies are under way

to isolate it and identify its function. In addition to genetic studies directed toward identifying markers for diabetogenic genes, Dr. Bell's laboratory employs molecular biology to study insulin secretion. These two complementary approaches are leading to a better understanding of the cause of diabetes mellitus that will ultimately result in new approaches for treating this disorder and for identifying individuals at increased risk.

Associate Investigator Stephen T. Reeders, M.D. (Yale University) and his colleagues have used the techniques of molecular genetics to gain insight into the underlying mechanisms of human hereditary renal disease. They have identified a number of genes that are candidates for sites for mutations in autosomal dominant polycystic kidney disease. In addition, two novel basement membrane collagen genes of potential importance in hereditary human nephritis have been isolated. One is the site for autoantibody binding in Goodpasture syndrome, a rare autoimmune disorder. These collagen genes also appear to be mutated in some cases of Alport syndrome.

Phenylketonuria (PKU), an inherited disease that causes severe mental retardation in children who lack a metabolic enzyme (phenylalanine hydroxylase, or PAH) in the liver, has been studied extensively by Investigator Savio L. C. Woo, Ph.D. (Baylor College of Medicine) and his colleagues. The procedure for genetic analysis in prenatal diagnosis of PKU was improved by the identification of additional polymorphic markers in the human PAH gene. Mutations in the PAH gene that are responsible for the disease were determined. The defective genes were found to have originated in different regions in Europe and Asia and then spread independently through prehistoric population migration. In addition, methods for directly introducing functional genes into the liver of animals were developed, and the simple procedure is expected to be useful for future correction of PKU and other liver deficiencies in humans.

Investigator David M. Kurnit, M.D., Ph.D. (University of Michigan) and his colleagues have found that the vast majority of nondisjunction errors are maternal and that deficient crossing over occurs in a plurality of Down syndrome offspring. By correlating maternal age with the type of nondisjunction, the group expects to determine the basis for the advanced maternal age effect on nondisjunction. A fluorescent detection system to monitor molecular polymorphisms will be employed to accomplish this analysis on a collection of four pericentromeric polymorphisms. A recombination-based assay to accomplish the isolation of genes on chromosome 21

has been developed that will assist in an efficient transcriptional analysis of chromosome 21. This protocol was used to demonstrate that the gene responsible for the fragile X syndrome is widely transcribed during fetal life.

The laboratory of Assistant Investigator Fred D. Ledley, M.D. (Baylor College of Medicine) is exploring the potential of somatic gene therapy by developing methods of potential application to methylmalonic acidemia (a model inborn error of metabolism), congenital hypothyroidism, and arthritis. The group has described two novel targets for gene therapy involving direct introduction of DNA into tissues.

The research activities of the laboratory of Investigator Stuart H. Orkin, M.D. (Children's Hospital, Boston) center on the molecular biology and genetics of blood cells. Mechanisms controlling gene expression and development of both red and white blood cells are under study. Genetic experiments have demonstrated that erythroid cell development is dependent on a single major transcription regulatory protein. A gene transfer rescue assay has been established that permits more refined study of the role of this protein in red cell development. Through study of a white blood cell–expressed cytochrome subunit, a repressor of gene expression has been identified. This protein (CDP) resembles a *Drosophila* protein (encoded by the *cut* gene) and, as such, highlights the extensive parallels of gene regulation across species boundaries.

The laboratory of Investigator Stephen A. Liebhaber, M.D. (University of Pennsylvania) studies the human hemoglobin and growth hormone genes, with the goal of understanding how specific members of these two gene families are expressed at particular times during fetal development and how their expression is limited to particular tissues (the red cells in the case of the globin genes and the pituitary and placenta with growth hormone genes). Answers to these questions will contribute to a more detailed understanding of gene function in normal individuals, as well as clarify defects underlying a broad range of genetic diseases.

The laboratory of Assistant Investigator Jane M. Gitschier, Ph.D. (University of California, San Francisco) studies the molecular basis of a variety of sex-linked disorders, including hemophilia A. A number of genes have been isolated and are being examined for their role in several of the inherited disorders. One appears to code for a copper transport protein and is defective in patients with the sex-linked disorder, Menkes syndrome. In a separate line of experimentation, the gene coding for an antidiuretic hormone in the kidney was found to be mutated in

several unrelated individuals with the kidney defect of nephrogenic diabetes insipidus. A mouse model of hemophilia also is being pursued for future gene therapy experimentation.

The laboratory of Investigator Yuet Wai Kan, M.D. (University of California, San Francisco) has been investigating the molecular basis of a group of hereditary disorders affecting the red cells. They have studied the mutations that give rise to hemoglobinopathies and thalassemia and devised simple, rapid, and nonradioactive tests for these mutations, which will facilitate DNA diagnosis in regions of the world where these diseases are common. The group has initiated cooperative studies in the Mediterranean area and in China. They also are investigating the control of globin gene expression and are exploring the protein-DNA interactions in the globin gene regions that are important for the high-level expression of globin in the erythroid cell, and the developmental switch from embryonic to fetal to adult hemoglobin. Sequences critical for the high level of globin gene expression have been identified and utilized to enhance globin gene expression in retrovirus-mediated gene transfer experiments. Such work may lead to the correction of the β-globin defects in sickle cell anemia and thalassemia. In addition, examination of the mechanism by which butyrates increase fetal hemoglobin gene expression may also prove useful for treatment of the β-globin defect.

The laboratory of Assistant Investigator David A. Williams, M.D. (Indiana University) studies factors that control the behavior of cells in the bone marrow, leading to the formation of all blood cells. Such cells, termed hematopoietic stem cells, represent an important component of the blood system for normal blood cell production and are important in some forms of leukemia. These cells are also the target cell population for gene modification in so-called somatic gene therapy. Using molecular and cell biologic methods, Dr. Williams's laboratory has identified and studied several new proteins important in the production of blood cells in the bone marrow cavity. The group has now demonstrated an ability to produce mice that express human proteins through the use of genetically modified bone marrow cells.

The laboratory of Assistant Investigator John W. Belmont, M.D., Ph.D. (Baylor College of Medicine) is studying how blood cell formation is controlled by the activity of the most primitive of blood cell precursors, the hematopoietic stem cells. Multiple approaches, including new tissue culture methods, introduction of marker genes, and gene cloning are being employed to analyze how these cells are committed either to undergo massive proliferation or to self-renew (make more stem cells). The long-term objective is to use this information in the treatment of human diseases by selective genetic alteration of these cells. In a second project, Dr. Belmont's group wishes to identify the gene responsible for Bruton's X-linked agammaglobulinemia, an inherited disease in which young males are unable to form antibodies because of a failure to develop B lymphocytes. The search for the gene has been narrowed to ~1/1,000th of the human genome. The laboratory has also developed a new carrier test for this disorder.

The research program of Associate Investigator David Ginsburg, M.D. (University of Michigan) and his colleagues focuses on the biology of the human blood-clotting system. The group has identified the molecular defects responsible for many forms of von Willebrand disease, the most common inherited bleeding disorder. The laboratory has continued to study plasminogen activator inhibitor-1 (PAI-1) and has characterized the genetic defect in a new human bleeding disorder due to a defect in the PAI-1 gene. Analysis of genetically engineered variant PAI-1 molecules has shed new light on the function of this important blood-clotting protein. These studies may eventually lead to new strategies for the treatment of blood-clotting disorders, including heart attack and stroke. Finally, in a new program attempts are under way to identify the genes responsible for graft-versus-host disease, the major complication of bone marrow transplantation.

Familial hypertrophic cardiomyopathy (FHC) is a primary heart muscle disorder, inherited as an autosomal dominant trait characterized by inappropriate myocardial hypertrophy and early mortality. During the past year, Investigator Jonathan G. Seidman, Ph.D. (Harvard Medical School) and his colleagues used a rapid screening method involving ribonuclease protection assays to identify cardiac myosin heavy-chain missense mutations in the genomes of FHC probands. They have now identified 11 different missense mutations, all in the head or head/rod junction portions of the cardiac myosin heavy chain. Most of these mutations involve a charge change in the encoded amino acid. They have also demonstrated that *de novo* arising missense mutations in β-cardiac myosin heavy chains can cause sporadic hypertrophic cardiomyopathy in patients whose parents are not affected. Surprisingly, individuals with certain mutations have a significantly shorter life expectancy than others, despite the fact that their clinical symptoms are otherwise indistinguishable. In many instances FHC cannot be diagnosed until patients have passed the growth spurt associated

with puberty. Genetic analyses of the children of FHC probands has confirmed the difficulty of clinical diagnosis of FHC in children. Approximately 50% of FHC in North America results from β-cardiac myosin heavy-chain missense mutations, and early genetic diagnosis will be useful to patients and physicians.

The research carried out in the laboratory of Investigator Bernardo Nadal-Ginard, M.D., Ph.D. (Children's Hospital, Boston) is directed toward understanding how the contractile apparatus of mammalian cells is produced and regulated. These cells and their contractile apparatus are responsible for the structure and function of skeletal muscle and the cardiovascular system. First, the group seeks to identify and characterize genes responsible for the activation and repression of other genes that are responsible for the production of the building blocks of the contractile system, the contractile proteins. Second, they wish to understand why muscle cells have lost the ability to divide and, therefore, to regenerate after an injury such as a myocardial infarction. Third, they are exploring the basic mechanisms that allow a single gene to produce several different proteins that, in many cases, can have quite different functional properties.

MyoD activates what appears to be the entire program for muscle cell differentiation when its expression is experimentally forced in a number of cell types. Investigator Harold M. Weintraub, M.D., Ph.D. (Fred Hutchinson Cancer Research Center) and his colleagues have focused on how MyoD activates the hundreds to thousands of genes responsible for making a muscle cell, and how MyoD is activated during development. They have obtained evidence for a new type of regulatory element—a protein that recognizes that MyoD has bound to the proper DNA site by interacting with both the MyoD protein and the MyoD DNA-binding site. The group will continue their studies of MyoD expression in development of frogs and worms, focusing on classical embryological approaches in the former and genetic approaches in the latter. In frog development, MyoD transcription is first activated throughout the entire blastula, only to be stabilized in presumptive mesoderm when induction occurs. In worms, MyoD is activated when a cell first becomes committed solely to the muscle lineage, and this requires only 500 base pairs of the upstream MyoD control region.

The laboratory of Associate Investigator Rebecca A. Taub, M.D. (University of Pennsylvania) is studying the molecular biology of liver growth by characterizing the response as the liver initiates regeneration immediately after partial hepatectomy. Under study are a particular growth factor–like molecule that may play a major role in coordinating the growth of multiple hepatic cell types during liver development and regeneration, proteins that regulate the genes expressed in regenerating liver, and proteins that regulate signals transmitted in hepatic cells.

Senior Investigator Philip Leder, M.D. (Harvard Medical School) and his colleagues have directed their efforts toward understanding malignant disease at the genetic level. In studies that often involve the use of transgenic (or genetically engineered) mice, the group examines the action of genes not just in cells, but in the organism as a whole. Recent work has focused on factors that modify tumorigenesis by either provoking or inhibiting tumor formation. Particularly important have been discoveries regarding the potent antitumor effects of a biologically active product of the immune system, interleukin-4. These studies have also shed light on the fundamental process of development in the mammalian embryo and the developing immune system.

The aim of the laboratory of Associate Investigator Andrew P. Feinberg, M.D., M.P.H. (University of Michigan) is to identify tumor suppressor genes on human chromosome 11. The group has found that at least two genes on chromosomal band 11p13 are involved in Wilms' tumor (WT), a childhood kidney cancer. They have shown the existence of a second WT gene on band 11p15, which appears to be involved in many common adult cancers. Also localized to this band is the hereditary disorder Beckwith-Wiedemann syndrome, which causes Wilms' and other childhood cancers. A novel strategy for identifying and isolating tumor suppressor genes has been developed that may have application to other areas of genetics, including efforts to clone genes involved in cellular aging. Through this new approach a subchromosomal fragment from 11p15 has been isolated, which causes cessation of growth when transferred into tumor cells.

The laboratory of Assistant Investigator Vikas P. Sukhatme, M.D., Ph.D. (University of Chicago) studies transcription factors in two different contexts. They have isolated and characterized genes induced by mitogenic signals. Some of these genes are stimulated in every mammalian cell type tested. Importantly, the proteins encoded by these genes encode transcription factors containing zinc finger motifs, thereby regulating transcription of a distal set of genes. These proteins therefore serve as nuclear signal transducers. Their expression is modulated in a wide range of biological processes in addition to cellular proliferation. Recently, Dr. Sukhatme's laboratory has identified similar genes expressed in the

kidney. One initially isolated by others is involved in the genesis of the embryonic kidney neoplasm Wilms' tumor. In collaboration with Dr. Frank Rauscher's laboratory at the Wistar Institute, it has been shown that the product of this gene functions to shut down other genes. Two growth factor genes have been identified as targets. These data provide novel insights into how Wilms' tumors arise and improve our understanding of kidney development.

Differentiated cells express unique sets of proteins that are located at different sites in the genome. A major interest of the laboratory of Associate Investigator Gary J. Nabel, M.D., Ph.D. (University of Michigan) is to understand how cells regulate the expression of these diverse sets of genes during development and following viral infection. To address this question, the T lymphocytes that protect the body from invasion by foreign organisms have been analyzed. These cells are also the major target of infection by the human immunodeficiency virus (HIV), which causes the acquired immune deficiency syndrome (AIDS). In T cells that contain the AIDS virus, this activation process is used by HIV to activate viral replication, and Dr. Nabel's group has cloned and is characterizing one of the genes encoding a protein that binds to control regions regulating the expression of HIV and other immune system proteins. Knowledge derived from the study of such regulatory proteins has been used to deliver recombinant genes into cells for further understanding and treatment of human diseases.

Research in the laboratory of Assistant Investigator Patrick O. Brown, M.D., Ph.D. (Stanford University) has focused primarily on biochemical mechanisms in the replication of retroviruses, including HIV, the AIDS virus. Purification of integrase, the enzyme that mediates the introduction of retroviral genes into the host cell's DNA, has facilitated the group's efforts to understand this essential step in retroviral replication. Studies of the purified enzyme are moving toward an understanding of the enzyme's structure and the mechanism by which it recognizes and recombines its two DNA substrates. In a separate area of investigation, Dr. Brown's group has recently developed a highly efficient new method for gene mapping, called genomic mismatch scanning. The method has been tested successfully using Baker's yeast as a test system. Current efforts are devoted to adapting the procedure so that it can be used to map genes that govern complex human traits.

The pathogenic human retrovirus HIV-1 (human immunodeficiency virus type 1) encodes two nuclear regulatory proteins, Tat and Rev, that are essential for viral replication in culture. Both Tat and Rev are known to regulate viral gene expression through direct interactions with structured viral RNA target sites termed, respectively, TAR and RRE. Over the past year the laboratory of Associate Investigator Bryan R. Cullen, Ph.D. (Duke University) has shown that the primary binding site for Rev on the RRE consists of as few as 13 nucleotides. Substitution of this minimal Rev-binding site in place of the viral TAR element was shown to permit Tat function in the context of a Tat-Rev fusion protein but precluded binding, and hence trans-activation, by wild-type Tat. Mutational analysis of both Tat and Rev in the context of this active fusion protein has facilitated the identification of the specific biological roles of the different essential subdomains present in these viral trans-activators. This functional dissection has clarified the mechanism of action of these critical HIV-1 regulatory proteins and should facilitate the design of potential inhibitors.

Research of Assistant Investigator Laimonis A. Laimins, Ph.D. (University of Chicago) and his colleagues centers on the molecular biology of human papillomavirus (HPV) types 16, 18, and 31, which have been implicated as the causative agents of cancers of the anogenital tract. Transformation studies *in vitro* have shown that both the E6 and E7 gene products are required for high-frequency transformation of keratinocytes, as assayed by an ability to immortalize and alter differentiation of human epithelial cells. In a system where epithelial cells are grown at an air-to-liquid interface, HPV sequences have been shown to induce morphological changes similar to those seen in genital intraepithelial neoplasias *in vivo*. Production of HPV has recently been induced from a continuous cell line concomitant with epithelial differentiation. Dr. Laimins and his colleagues have also examined in detail the transcriptional regulatory mechanisms utilized by papillomaviruses. One viral enhancer has been shown to be responsible for the tissue-specific expression of HPV genes and has been found to be regulated entirely by cellular factors. Factors controlling this enhancer include members of the ubiquitous AP-1 family, as well as a novel transcription factor, KRF-1. Competition between KRF-1 and Oct-1 for binding has been observed and may reflect a mechanism for repression of HPV expression.

The research efforts in the laboratory of Assistant Investigator John B. Lowe, M.D. (University of Michigan) focus on understanding the function and regulation of specific mammalian cell surface carbohydrate molecules and the glycosyltransferase genes that determine their expression. Several such genes have been isolated and characterized using systems developed in this laboratory. These genes and sys-

tems have been used to identify carbohydrate molecules that allow specific immune cells to leave the blood circulation and thus promote their participation in the inflammation process. More recently, Dr. Lowe and his colleagues have used these genes to construct such carbohydrates and have found that these molecules function as anti-inflammatory compounds in animal models of inflammation. The genes are also in use in studies designed to answer other questions about the regulation and function(s) of carbohydrate molecules in mammalian organisms.

Associate Investigator Stephen V. Desiderio, M.D., Ph.D. (Johns Hopkins University) and his colleagues continue to study development and activation of the immune system. Production of antibodies depends on a series of DNA rearrangements that occur in developing immune cells. These rearrangements are triggered by the RAG-1 and RAG-2 proteins. The laboratory has pinpointed specific chemical modifications that regulate the activity of RAG-2 and its level in the cell. These observations suggest that DNA rearrangement and control of cell division may be coupled in developing immune cells. Dr. Desiderio also continues to study how an immune response is triggered. Having identified novel signal-transducing proteins expressed in immune cells, his laboratory has begun to define other molecules that interact specifically with these proteins, with the ultimate goal of linking events at the cell surface to responses that occur in the nucleus.

Associate Investigator Thomas R. Kadesch, Ph.D. (University of Pennsylvania) and his colleagues have continued to work on the immunoglobulin heavy-chain enhancer in B cells and have begun to address transcriptional control mechanisms in other hematopoietic cell types. The laboratory has continued to characterize a candidate-repressor protein, Zeb, and to explore the role of another inhibitor, Id, in the context of B cell development. Id also appears to play a role in the development of myeloid cells. Thus B cells and myeloid cells employ a common family of transcription factors to control their developmental maturation and the expression of their genes.

The activity of antibody genes in B cells has been proposed to be regulated by a B cell–specific activator protein, Oct-2. The laboratory of Assistant Investigator Harinder Singh, Ph.D. (University of Chicago) has used a gene-targeting procedure to reduce severely the levels of Oct-2 in a B cell. Surprisingly, the activity of antibody genes was unaffected in the mutant cells. This result strongly suggests that B cells can use a different pathway involving the non-cell-type–specific, but related, activator protein

Oct-1 to regulate the activity of B cell–specific immunoglobulin genes. The Oct-2 mutant cells are, however, defective in activating transcription of certain constructed genes. This result suggests that Oct-2 is critically required to regulate the activity of B cell genes that have yet to be identified.

Histocompatibility molecules are responsible for transplant rejection and the binding and presentation of viral, bacterial, and tumor antigens to the immune system. To bind a large number of these antigens and insure the survival of the species it is beneficial that many varieties of the histocompatibility molecules be found in the population. The microrecombination process, which generates variety by reassorting genetic information among histocompatibility and related genes in germ cells, has been previously studied by isolating mutant mice that differed from their parents in histocompatibility genes. However, to study this process, Assistant Investigator Jan Geliebter, Ph.D. (Rockefeller University) and his colleagues are using a reporter construct that turns microrecombinant cells blue under specific conditions, and they are introducing this construct into mammalian cells in culture and transgenic mice.

The parasites that cause the major tropical diseases of the world possess sophisticated molecular mechanisms for evading the immune response mounted against them. The laboratory of Investigator John E. Donelson, Ph.D. (University of Iowa) seeks to understand these mechanisms and devise strategies for combating them. African trypanosomes, for example, evade the immune response by periodically switching the major protein on their surface. The molecular events associated with this switch are being investigated. *Leishmania* parasites, on the other hand, possess on their surface three forms of a protease that enable them to penetrate and survive within macrophages. The differential expression and biological functions of these three forms are being examined.

Investigator Mario R. Capecchi, Ph.D. (University of Utah) and his colleagues have developed technology that allows an investigator to mutate specifically any gene in the mouse. This technology was used by the group to disrupt genes believed to have important roles in establishing positional information along the anterior-posterior axis of the early mammalian embryo. Where should the heart develop? Where should specific nerves develop? How is the shape of the face determined? Disruption of some of these genes resulted in mice with heart defects, loss of specific cranial nerves, or craniofacial abnormalities. When this approach is used to study mammalian development, a correlation is established

between loss of a specific gene with specific phenotypic consequences, and this thereby establishes the function of that gene in development. By comparing the phenotypic consequences of mutating a series of genes in a developmental network, it is hoped that a logic of how that network functions will emerge.

The laboratory of Investigator Shirley M. Tilghman, Ph.D. (Princeton University) is also studying the genes responsible for appropriate development of the mouse. This year her laboratory proposed a model to explain how two neighboring genes could influence each other's expression by competing for a common set of regulatory elements. The consequence of the competition is that only one of the two genes is expressed from a single chromosome, a phenomenon known as parental imprinting. Her laboratory also demonstrated that the repression of the α-fetoprotein gene in liver is a complex process, in that the regulatory elements necessary for repression in hepatocytes around the central vein are different from those in the intermediate and periportal hepatocytes. The laboratory is using classical genetics to map two genes in which mutations cause defects in development. Mutations in the *Fused* gene result in an overgrowth of neuroectoderm, whereas *piebald* mutations affect the development of two neural crest lineages, the melanocytes and enteric ganglia. The genetic analyses will be important in strategies to clone these genes.

Research in the laboratory of Assistant Investigator Philippe M. Soriano, Ph.D. (Baylor College of Medicine) continues to concern developmental genetics in mice. They have produced mutant mice for several nonreceptor tyrosine kinases, using gene-targeting techniques in embryonic stem cells. These kinases play essential roles in transducing signals from receptors with extracellular domains. Whereas disruption of the *src* gene in mice leads to the bone disease osteopetrosis, disruption of other related kinase genes does not lead to overt phenotypes. Studies of the fyn^- mice have revealed defects in signaling from the T cell receptor and in the hippocampus, leading to defects in the immune system and in learning and memory, respectively. Crosses between kinase mutant mice lead to novel phenotypes, embryonic death or glomerulonephritis in the case of animals deficient for both *yes* and *fyn*. Another aspect of Dr. Soriano's work involves the generation of mouse mutations using "promoter traps," in which the gene is both mutated and its activity traced by expression of an introduced promoterless reporter gene. These studies have led to the identification of 18 new recessive lethal strains, of which one is a mutation in a broadly expressed transcription factor, TEF1.

The laboratory of Investigator Richard D. Palmiter, Ph.D. (University of Washington), in collaboration with Dr. Ralph Brinster at the University of Pennsylvania, uses gene transfer into the germline of mice to study various aspects of development and disease. The genes of interest are injected into pronuclei of fertilized eggs, where they integrate into one of the mouse chromosomes and become new genetic traits. By combining the regulatory regions (promoters and enhancers) from one gene with the region that encodes a particular protein from another, it is possible to direct the expression of novel gene products to specific cell types. This approach is currently being used to study the development of the mammalian nervous system. For example, the expression of nerve growth factor was directed to sympathetic neurons using the promoter/enhancer from a gene required for synthesis of their neurotransmitter, norepinephrine. This showed that growth factor gradients are not required to guide sympathetic axons to their targets, but are required to establish the normal pattern of innervation within the target tissue. The small population of neurons that normally use epinephrine as their neurotransmitter have been eliminated from a line of mice, by expressing diphtheria toxin using the promoter/enhancer from a gene involved in epinephrine synthesis. Studies of these mice now provide a means for ascertaining normal neuron function. Methods are also being devised to alter the neurotransmitters that neurons normally make to explore further the effects of these molecules.

The laboratory of Assistant Investigator Gregory S. Barsh, M.D., Ph.D. (Stanford University) is studying the mouse *agouti* (A) locus. Some dominant A mutations (A^y, A^{vy}) produce obesity and liver tumors in the animals, and some recessive mutations (a^x) are lethal during early embryogenesis. A candidate gene for A, ASP, has been isolated that appears to encode a small secreted protein, and a cell culture system has been developed to study increased tumor susceptibility mediated by A^{vy}. Molecular alterations have been identified in A^y and in a^x that appear to affect the expression but not the structure of ASP. Studies are currently under way to determine if and how different *agouti* mutations affect pigment synthesis, obesity, tumor formation, and early development.

The goal of the laboratory of Assistant Investigator Jeffrey M. Friedman, M.D., Ph.D. (Rockefeller University) is to clone genes that, when defective, give rise to obesity. Extensive studies of human twins have suggested that body weight in a given individual is physiologically regulated and that each person's weight is determined in part by a set of specific

genes. New technologies in molecular genetics have made it feasible to isolate these genes, and the laboratory is attempting to clone two such genes: the mouse obesity genes *ob* (*obese*) and *db* (*diabetes*). Each gene, if defective, results in a grossly obese state, with affected mice weighing up to three times normal. Recent progress has narrowed the search for the *ob* gene to ~400–600 base pairs of DNA on mouse chromosome 6. Similar efforts have localized *db* to a relatively small region of mouse chromosome 4. The identification of these genes should lead to a fuller understanding of the factors that control body weight in health and disease.

The laboratory of Assistant Investigator Paul A. Overbeek, Ph.D., M.B.A. (Baylor College of Medicine) is studying a transgenic family of mice (OVE210) with a mutation that causes a reversal of the normal left-right polarity of embryonic development. Homozygous mice show a transposition of their internal organs, with their stomach, spleen, pancreas, and heart all located on the right rather than the left side of their body. This condition, *situs inversus*, also occurs occasionally in humans. Since the mutation in these mice is linked to transgenic DNA, recombinant DNA techniques have been used to isolate the adjacent genomic sequences. These sequences map to mouse chromosome 4, in a region homologous to human chromosome 9. This novel *situs inversus* mutation may allow the first positional cloning of a gene that specifies polarity during normal mammalian embryogenesis.

Mutations that disrupt the development and behavior of nematodes have been analyzed to define the "genetic flowcharts" that control normal development and behavior. The laboratory of Associate Investigator Paul W. Sternberg, Ph.D. (California Institute of Technology) has found four genes similar to mammalian oncogenes that act in series to control the fates of cells during development of the male and hermaphrodite reproductive system of the nematode. Other genes that act antagonistically to this set of four genes have been identified. Neurons controlling various steps in male mating behavior have been identified by killing individual cells and observing subsequent defects in mating.

Assistant Investigator Carl S. Thummel, Ph.D. (University of Utah) and his colleagues are studying the molecular basis of metamorphosis in the fruit fly *Drosophila melanogaster* by isolating and characterizing regulatory genes induced directly by the steroid hormone ecdysone. One set of regulatory genes is induced by a relatively low ecdysone concentration 1–2 days before the onset of metamorphosis. The proteins encoded by these genes comprise the transcriptional machinery required for the subse-

quent induction of a second wave of regulatory gene activity, with the peak hormone titers that precede puparium formation. This second set of genes both transduces and amplifies the hormonal signal by coordinating the induction of large sets of secondary-response genes. Further studies of the genetic circuitry activated by ecdysone should provide key insights into the mechanisms of steroid hormone function in higher organisms, as well as provide a paradigm for the control of genetic regulatory hierarchies during development.

The laboratory of Investigator Thomas C. Kaufman, Ph.D. (Indiana University) is involved in the analysis of the homeotic genes of *Drosophila*. These genes act as developmental switches that direct the synthesis of DNA-binding proteins and regulate the expression of other genes. Moreover, these control genes are present and perform similar functions in flies and mice. The group has shown that the spatial patterns of expression of these genes are in separately regulated subdomains. This subregulation is seen in the portions of the DNA of each gene responsible for the subdomains, as well as in the identity of other genes that regulate each homeotic gene in its proper pattern.

Assistant Investigator Shigeru Sakonju, Ph.D. (University of Utah) and his colleagues are studying the molecular mechanisms by which homeotic genes specify identities of body segments in *Drosophila*. The research focuses on three homeotic genes, called *Ubx*, *abd-A*, and *Abd-B*, that specify the fate of cells in thoracic and abdominal segments, and on downstream genes that are regulated by them. The laboratory's experiments have shown that the homeotic proteins use cell-type–specific mechanisms to regulate the expression of a downstream gene. The work has also revealed that the identities of body segments are not determined at the same time during embryonic development. Rather, the identities of abdominal segments are established prior to those of thoracic segments.

The mechanisms by which the segment polarity and homeotic genes of *Drosophila* control segmental differentiation are the focus of the laboratory of Assistant Investigator Philip A. Beachy, Ph.D. (Johns Hopkins University). His group has isolated and characterized at the molecular level the segment polarity gene *hedgehog*, which encodes a protein targeted to the secretory pathway. Expression of *hedgehog* functions in, and is sensitive to, cell-cell signaling for the purpose of specifying positional identities of cells within segments. Additional progress was made in the determination and comparison of DNA-binding specificities for homeodomains encoded by several homeotic genes, as a step toward

understanding the basis for functional specificity of these genes. A gene encoding a novel homeodomain also was identified as a target for regulation by the homeotic gene of the bithorax complex.

Genetic analysis of *Drosophila* has identified most of the genes involved in early development. Many of these genes contain a homeodomain (HD), a DNA-binding motif also present in mammals. The laboratory of Associate Investigator Claude Desplan, Ph.D. (Rockefeller University) is studying how proteins with related sequences, such as the HD, perform their specific roles during development. The determinants of HD specificity *in vivo* are being investigated. Single–amino acid changes can affect both the base pair recognition and the dimerization state of HD proteins and therefore affect their function during development. Other DNA-binding domains such as the Paired domain are also found associated with the HD and act in combination or independently to broaden the specificity range of the developmental protein. Another level of specificity is achieved by post-transcriptional control of the HD proteins. For example, the activity of the Bicoid morphogen, whose concentration gradient is required for anterior patterning, is modified by a signal transduction pathway at the anterior pole of the embryo, leading to its inactivation. The nature of these modifications is being investigated both *in vivo* and biochemically.

Position-effect variegation is a phenomenon seen in *Drosophila* in which a chromosome rearrangement places a gene in an unaccustomed location, leading to its dysfunction. For one particular gene, the copy on the normal homologous chromosome also becomes dysfunctional. The laboratory of Investigator Steven Henikoff, Ph.D. (Fred Hutchinson Cancer Research Center) is investigating the genetic basis for this effect on the homologue. In other work, they are studying a chromosome that is highly unstable throughout development, apparently due to a chromosomal rearrangement that causes centromere dysfunction. Methods for detecting distant relationships between sequences of proteins are also under development.

Assistant Investigator Norbert Perrimon, Ph.D. (Harvard Medical School) and his colleagues have undertaken a molecular and genetic analysis of two signal transduction pathways implicated in cell fate determination in the *Drosophila* embryo. The first pathway operates through local activation of the receptor tyrosine kinase *torso* gene. Work has shown that the *D-raf* serine/threonine kinase, a *csw* nonreceptor protein-tyrosine phosphatase, and *ras-1* are required to transduce the *torso* signal. The second pathway under investigation involves the secreted protein of the *wingless* gene, which shows significant homology to the mammalian *Wnt-1* protein. Among the signal transducers isolated so far are a *zw3* serine/threonine kinase and a novel *dsh* protein. The long-term goal of the laboratory is to identify the components involved in these pathways and test their interactions in order to decipher how the morphogenetic signals from these systems control gene expression in the receiving cells.

Through enhancer detector screens, two *Drosophila* genes that are expressed in the differentiating embryonic peripheral nervous system (PNS) have been cloned and sequenced in the laboratory of Assistant Investigator Hugo J. Bellen, D.V.M., Ph.D. (Baylor College of Medicine). Both genes are expressed in the sensory motor cells and differentiating PNS. One gene encodes a protein with an RNA-binding domain that may regulate RNA processing of genes required for normal functioning of the PNS. The other gene encodes an extracellular immunoglobulin-like molecule that is membrane-associated and secreted. This gene may play a role in the fasciculation of peripheral neurons and formation of the neuromuscular junction. Also under investigation is a third gene that encodes synaptotagmin, a synaptic vesicle protein. Studies on its role in the release of neurotransmitters suggest that it is an essential gene.

Research in the laboratory of Assistant Investigator Stephen M. Cohen, Ph.D. (Baylor College of Medicine) is concerned with the origin and control of limb development during embryogenesis. This problem is particularly accessible in the *Drosophila* because of the ease of isolation of mutations that disrupt normal developmental processes. These studies have led to identification of a key gene that controls limb development in the *Drosophila* embryo. The *Distal-less* gene is required to instruct embryonic cells to become a part of the prospective limb. This gene is expressed in the presumptive limb cells and may initiate their developmental program by activating a set of genes specific for limb development. It is therefore important for the embryo to regulate stringently the locations at which the gene is activated. The means by which this control is effected are being studied. Genes through which *Distal-less* acts are also being explored in order to understand the organization of limb development at a molecular level.

Investigator Michael Rosbash, Ph.D. (Brandeis University) and his colleagues work in two different areas. They are examining gene expression with an emphasis on the mechanisms by which RNA transcripts are processed in the nucleus before they are transported to the cytoplasm for protein synthesis.

This work is done in yeast, because of its genetic tractability. Their second interest concerns the biochemical nature of the biological clock that drives daily changes in behavior and physiology. The *Drosophila* is their model system, because the fruit flies manifest circadian rhythms, and mutant strains are available that have aberrant or absent rhythms.

The laboratory of Investigator Michael W. Young, Ph.D. (Rockefeller University) also employs *Drosophila* as a model system to study mechanisms of circadian rhythms and developmental pathways. Mutations of the *period* (*per*) gene influence the fly's circadian rhythms. *In vitro* mutagenesis reveals a region of ~20 amino acids where substitutions predominantly generate short period phenotypes. Possibly, this region of the protein suppresses activity of the *per* protein—and therefore plays a regulatory role—in wild-type flies. A second chromosome being studied is *timeless* (*tim*), which produces arrhythmia for both eclosion and locomotor activity like *per*. It also affects expression of *per*, altering its circadian cycling. In other work, neurogenic genes essential for correct patterns of mesodermal development are under scrutiny. Seven genes may work together to influence cell fate choices undertaken independently in mesoderm and ectoderm. Investigation of the molecular details underlying genetic interactions between the *Notch* and *Delta* genes has shown that single–amino acid substitutions measurably alter interactions between their proteins, which may change intracellular signaling by the *Notch* protein.

The main research interest of Investigator Robert Tjian, Ph.D. (University of California, Berkeley) and his colleagues concerns the means by which the genetic information stored in DNA is retrieved in a controlled and orderly fashion during the biochemical process of transcription, which subsequently leads to the expression of specific genes in animal cells. The laboratory has taken a biochemical approach to the problem of gene control and has devised various means of isolating the individual cellular components responsible for transcription and of reconstructing this complex reaction in the test tube. In this way, they can study how specific genes are turned on and off during cell growth and development of eukaryotic organisms. The mechanisms that govern the switching on and off of genes are of fundamental importance in understanding the normal metabolic processes that maintain and perpetuate living cells, as well as in deciphering the basis of disease and other cellular or genetic disorders.

The alteration of cellular phenotype, as might occur during oncogenesis or cell differentiation, is largely a function of alterations in the control of gene expression. The basis for such control has been investigated by the laboratory of Investigator Joseph R. Nevins, Ph.D. (Duke University) in systems that focus on transcriptional regulatory mechanisms, particularly as they relate to oncogenic transformation by the products of DNA tumor viruses. These studies have elucidated basic mechanisms for the control of transcription factor activity and have provided a unification of the manner in which viral oncoproteins disrupt the action of the retinoblastoma tumor suppressor protein Rb. Finally, similar strategies have focused on the underlying mechanisms for gene control via the processing of mRNA precursors to create polyadenylated 3' termini. This work has identified factors that control the processing of a pre-mRNA that are likely involved in the control of gene expression during differentiation of lymphocytes.

In a mouse fibroblast cell line, immediate-early transcription factors are induced within minutes after nonproliferating cells are exposed to growth factors. Within two or three hours, a set of delayed-early genes is activated, presumably by the induced transcription factors. A number of delayed-early genes have been identified by cDNA cloning and sequencing by the laboratory of Senior Investigator Daniel Nathans, M.D. (Johns Hopkins University). Such genes include those that encode a protein affecting the mobility of macrophages, a membrane protein involved in water transport, a DNA polymerase, an enzyme involved in purine nucleotide synthesis, nonhistone chromosomal proteins, and cyclin CYL1, which is thought to regulate an early step of the cell division cycle. Upstream of some of the genes are binding sites for immediate-early transcription factors. Studies are in progress to determine whether these genes are activated by the transcription factors.

New experiments on translational phenomena in the laboratory of Investigator Raymond F. Gesteland, Ph.D. (University of Utah) have revealed complexities that more and more point toward the crucial involvement of an intricate folding of messenger RNA molecules in regulating translation. Genetic experiments are beginning to define elements of the ribosome that recognize structural features in the folded mRNA. An unusual example is in the mRNA for gene *60* that has a 50-nucleotide region over which ribosomes hop very efficiently. Detailed features in the mRNA of retroviruses are sensed by ribosomes in order to circumvent a stop codon and to make the crucial reverse transcriptase. The inescapable conclusion from these experiments is that a

much deeper knowledge of structures in natural RNA molecules is required.

Chemical reactions necessary for life are typically catalyzed by large molecules called enzymes. These are usually proteins, but in some cases ribonucleic acid (RNA), a form of genetic material, acts as an enzyme. The detailed folding of the RNA chain that allows it to act as a catalyst has been a subject of much interest. In the past year the laboratory of Investigator Thomas R. Cech, Ph.D. (University of Colorado) converted one of the substrates bound by an RNA enzyme to an "explosive device." By identifying points of destruction of the RNA caused by this reagent, information about the three-dimensional structure of the active site was obtained. It has been thought that RNA enzymes catalyze an extremely limited repertoire of reactions. In the past year this group discovered that an RNA enzyme could catalyze a new class of reactions involving amino acids, the building blocks of proteins, rather than nucleotides, the building blocks of nucleic acids.

Investigator Joan A. Steitz, Ph.D. (Yale University) and her colleagues are investigating how a number of small particles found in all cells contribute to basic life processes. These particles contain RNA and protein, and play essential roles in the multiple steps by which the information in the cell's DNA is expressed in the form of proteins. For instance, several of these particles are involved in RNA splicing whereby nonsense segments are removed from the RNA copies of genes, converting them to functional messengers. Important tools used to study these small particles are antibodies made by some patients with rheumatic diseases, like systemic lupus erythematosus. Understanding the nature of the particles is therefore important not only to basic molecular biology but also for improving the diagnosis and treatment of rheumatic disease.

When cells of all types are exposed to environmental stress, such as mildly elevated temperatures, they respond by producing a small number of proteins called the heat-shock proteins. This response is one of the most highly conserved genetic regulatory systems known. The research of Investigator Susan L. Lindquist, Ph.D. (University of Chicago) and her colleagues focuses on two aspects of the response. First, the laboratory is investigating the specific molecular functions of the heat-shock proteins and has found that hsp82 is required for the formation of functional steroid receptors and plays an important role in activating the oncogenic protein pp60$^{v\text{-}src}$. On the other hand, hsp104 is required for cells to tolerate short-term exposure to extreme temperatures and other forms of stress without dying. This

highly conserved protein contains two nucleotide-binding sites, both of which are required for stress tolerance. Genetic experiments demonstrate that the protein is related in function to hsp70, another highly conserved protein. Second, the rapid and reproducible induction of new proteins provides a general model system to investigate mechanisms of genetic regulation in higher organisms. This year the group has examined a new mechanism for regulating hsp70, by sequestration. This was first discovered when tissue culture cells were forced to express the protein in the absence of stress. In normal *Drosophila* the mechanism operates in early embryos, where excess hsp70 expression may interfere with normal growth.

Recent studies indicate that in the living cell there are specialized proteins called chaperones that help newly made proteins adjust from unfolded forms into characteristic biologically active three-dimensional structures. The laboratory of Associate Investigator Arthur L. Horwich, M.D. (Yale University) originally discovered that heat-shock protein 60 is a chaperone inside mitochondria. The 14 hsp60 molecules comprise two stacked rings or a double-donut structure with which unfolded proteins become associated. In the presence of ATP and a second single-ring structure, proteins fold into the active forms and are released. During this past year the group has uncovered a new class of double-ring structures that also appear to be mediators of protein folding. One member was found in thermophilic archaebacteria, organisms that grow at temperatures near that of boiling water, and a second, related structure was detected in the cytoplasm of cells of higher organisms.

The laboratory of Associate Investigator Jeffry L. Corden, Ph.D. (Johns Hopkins University) has discovered an unusual extension or "tail" on RNA polymerase II, the enzyme that carries out the first step in expression of genetic information. The tail is a site of modification by enzymes that respond to signals sent from outside the cell. How these signals modify the tail and how these modifications affect gene expression are the focus of current experiments. The results of these studies should allow for a better understanding of the regulation of gene expression in normal processes such as growth and development and in abnormal situations such as cancer and birth defects.

The research of Investigator Maynard V. Olson, Ph.D. (Washington University) involves interrelated studies of yeast and human DNA. Because yeast and human cells have similar genetic and biochemical properties, information gained by studying yeast is

often readily transferable to human biology. Yeast has also proved to be an excellent host for the propagation of large segments of cloned human DNA. Dr. Olson's laboratory has developed a high-resolution map of the DNA normally present in yeast cells and is cooperating with other groups involved in yeast research to promote the use of such maps as major tools in genetics research. His laboratory is also continuing to develop applications of yeast-based cloning systems to human genetics. The use of sequence-tagged sites (STSs), landmarks within human DNA that can be completely described in an electronic database, has received particular attention. STS-based mapping of human DNA cloned into yeast is finding wide applications in the international effort to map the human genome. Finally, studies of the reintroduction of DNA into mammalian cells, following propagation in yeast, are under way; this experimental procedure offers a path to the functional analysis of the cloned DNA.

A major project of the laboratory of Assistant Investigator George M. Church, Ph.D. (Harvard Medical School) is the development of technologies for analysis of genomes in order to compare them for conserved and variable genetic elements. These technologies include nonradioactive chemiluminescent multiplex sequencing; REPLICA automated sequencing, reading, and comparison software; *in vivo* footprinting; and subtractive sequencing. These methods are being applied to the development and correlation of whole genome databases of DNA polymorphic sites, protein termini, protein abundances, and interactions among cellular components.

GREGORY S. BARSH, M.D., PH.D., *Assistant Investigator*

Genomic and Functional Characterization of the Mouse *agouti* (*A*) Locus

A major focus in Dr. Barsh's laboratory has been the positional cloning and analysis of preexisting mouse developmental mutations. In the past year, candidate cDNAs for the mouse *A* locus have been isolated, and molecular defects associated with two recessive lethal *A* mutations have been characterized.

Mice of *agouti* genotype *A/A* have hairs with a subapical band of pheomelanic (yellow) pigment on an otherwise eumelanic (black) background. In general, recessive *agouti* mutations produce a completely black hair and dominant *agouti* mutations produce a completely yellow hair. Some *agouti* mutations, such as a^x (*lethal non-agouti*), result in embryonic lethality when homozygous; the laboratory has previously shown that these recessive lethal genes represent three complementation groups. In addition, most dominant *agouti* mutations, such as A^y (*lethal yellow*) and A^{vy} (*viable yellow*), are associated with premature infertility, adult-onset obesity, increased somatic growth, and increased susceptibility to tumor formation. Transplantation studies suggest that the *agouti* gene acts in cells of dermal origin to control a signaling pathway that ultimately determines the type of pigment (pheomelanin or eumelanin) synthesized by follicular melanocytes.

To isolate the gene(s) affected by *agouti* mutations, Dr. Barsh has taken a systematic approach in which a physical map, based on the somatic cell genetic technique of radiation hybrid mapping, has been paired with the development of a genetic map, based on an interspecific backcross using the A^y and a^x alleles opposite a *Mus castaneus* chromosome (which carries the *A* allele). The group found that the genetic maps surrounding A^y and a^x are indistinguishable and that two closely linked proviral insertions, *Emv-15* and *Xmv-10*, are not responsible for the A^y and *a* mutations, respectively. The results also eliminated several candidate genes (phospholipase C, *Src*, phosphotyrosine phosphatase) from consideration as mediators of the tumor-promoting aspects of the A^y and A^{vy} alleles. (The work described above was supported, in part, by a grant from the National Center for Human Genome Research.)

The occurrence of a radiation-induced mutation doubly heterozygous for *limb deformity* (*ld*) and *extreme non-agouti* has provided a fortuitous breakthrough in Dr. Barsh's efforts to understand the molecular basis of *agouti* mutations. As described by Dr. Rick Woychik and his colleagues, this mutation is associated with a chromosomal inversion, Is1GsO, that allows the isolation of genomic sequences closely linked to the *A* gene. Dr. Barsh's laboratory used these genomic fragments to isolate two cDNAs that span the Is1GsO breakpoint and to investigate the structure of the A^y and a^x chromosomes. One cDNA detects a 900-bp mRNA expressed in the skin and testes of *A/A* mice and in many additional organs (brain, kidney, liver, spleen) of A^y/a mice. This cDNA has the potential to code for a protein of ~17 kDa that contains two potential amino-linked glycosylation sites and a putative amino-terminal signal sequence. Similarity searches using the predicted peptide sequence are not revealing. However, the predicted 5′-untranslated region contains an ~180-bp region that is nearly identical to the nontranscribed strand of a retrotransposon element, VL-30. Expression of this candidate *agouti* cDNA in the same cell with one or more VL-30 elements, which are developmentally regulated, is likely to result in the formation of double-stranded RNA. Thus it is possible that expression of the *agouti* gene is regulated, in part, via the production of natural antisense RNA. A second cDNA interrupted by the Is1GsO breakpoint is 2.1 kb in length and detects a 6-kb mRNA expressed in the testes of *A/A* and A^y/a mice. Coding regions of the 900-bp and the 2.1-kb cDNA are transcribed from opposite strands and are located within the same genomic fragment, but are spliced such that their exons are non-overlapping.

Using genomic cloning and mapping, this laboratory has determined that the A^y mutation is associated with a chromosomal rearrangement that affects the mobility of multiple high-molecular-weight restriction fragments centromere-proximal to the coding sequences of both the 900-bp and the 2.1-kb cDNA they have isolated. By contrast, the a^x mutation is associated with a deletion of at least 25 kb of DNA centromere-distal to cDNA-coding sequences. Dr. Barsh speculates that A^y and a^x affect *agouti* coat color by altering regulatory sequences 5′ and 3′, respectively, of the 900-bp cDNA, to produce either a gain (A^y) or a loss (a^x) of function.

To investigate the role of the cDNAs the laboratory has isolated in the increased tumor susceptibility observed in $A^{vy}/-$ and $A^y/-$ animals, they have, in collaboration with Drs. George Wolff and Wendy

Hsiao, shown that fibroblast cell lines derived from neonatal A^{vy}/a animals exhibit a remarkably high degree of spontaneous transformation in cell culture compared with congenic a/a animals. The transformed cells form foci in monolayer cultures, exhibit anchorage independence, and form tumors when injected into nude mice. In addition, transformed cells derived from A^{vy}/a fibroblasts always contain restriction fragment length variants (RFLVs) associated with the A^{vy}-containing chromosome but occasionally lose RFLVs associated with the a-containing chromosome. Furthermore, transfection of a/a fibroblasts with DNA from $A^{vy}/-$ fibroblasts confers the phenotype of increased spontaneous transformation, which suggests this is a dominant trait that may be analogous to the increased tumor susceptibility observed in $A^y/-$ or $A^{vy}/-$ animals *in vivo*. Expression of the 900-bp cDNA in a/a fibroblasts will help to determine whether the pleiotropic effects of A^y (and A^{vy}) are mediated by a single transcriptional unit.

Dr. Barsh is also Assistant Professor of Pediatrics at the Stanford University School of Medicine.

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GENETIC CONTROL OF SEGMENTAL DIFFERENTIATION

PHILIP A. BEACHY, PH.D., *Assistant Investigator*

The ontogeny of many animal groups entails subdivision of the embryo into a series of homologous segments to be differentiated along distinct pathways. These processes are best understood in *Drosophila*, where they are controlled by a temporally and spatially ordered hierarchy of gene expression. The work in Dr. Beachy's laboratory centers upon the two groups of genes within this hierarchy that are most directly concerned with control of segmental differentiation. The segment polarity group specifies the positional identities of cells within a segment, and the homeotic group specifies the distinct identities of individual segments. Both are required to produce the diverse array of segmentally specialized structures characteristic of the *Drosophila* larva and adult.

Identification of a New Signaling Molecule Required for Positional Identity

During the syncytial period of early *Drosophila* embryogenesis, morphogens (primarily transcriptional regulatory proteins) can exert effects upon gene expression in distant nuclei by diffusion from the sites at which they are synthesized. This form of position-dependent communication is required to coordinate subdivision of the embryo into broad zones and eventually into segments. Following the syncytial stage, further signaling activity functions to specify and refine the positional identities of cells within a segment. At this stage, however, signaling mechanisms must reckon with the presence of cell membranes.

Positional identities of cells within a segment are the province of the segment polarity genes. John Lee, Doris P. von Kessler, and Dr. Suki Parks have isolated and characterized at the molecular level a segment polarity gene whose mutant phenotype was first reported 12 years ago. This gene, named *hedgehog* (*hh*) for the continuous pattern of bristles observed on the ventral surface of mutant larvae, is required to maintain the positional identities of cells to either side of the parasegment boundary. This boundary marks an important cell lineage restriction and functions as a frame of reference for later expression of homeotic genes. Long before any overt morphological differentiation, the boundary is defined by the expression of the segment polarity genes *wingless* (*wg*) in a stripe of cells to the anterior of the boundary and *engrailed* (*en*) in a neighboring stripe of cells to the posterior. The *en* and *wg* genes are mutually required for maintenance of the other's expression; their expression in different

cells implies the existence of a cell-cell signaling pathway, with subsequent processing of the signals to affect gene expression in the nucleus.

The genetic evidence linking *hh* to this pathway is its requirement for the maintenance of *en* and *wg* expression. *In situ* hybridization to wild-type embryos demonstrated that the *hh* locus is expressed coincidently with *en* in posterior cells within each segment of the embryo. Similar studies with mutant embryos revealed that the establishment of *hh* expression requires input from earlier-acting pair-rule genes, while its maintenance requires normal function of other segment polarity genes, including *en* and *wg*. The *hh* gene thus functions in and is sensitive to positional signaling. Sequence analysis and *in vitro* translation studies suggest that the *hh* protein product is targeted to the secretory pathway. All of these results are consistent with a role for *hh* in signaling the *en* cell identity to neighboring cells, a model supported by various studies, including genetic mosaics that demonstrate a noncell autonomous requirement for *hh* in cuticle patterning.

Future work will draw upon the existing genetic and molecular studies of other *Drosophila* segment polarity genes to help identify the *hh* protein receptor or target and to characterize the intracellular consequences of signaling. Studies of other segment polarity genes also suggest that *hh* homologues may exist in other species, including mammals.

Segment Identity

Following the subdivision of the embryo into segments and the specification of positional identities within them, the homeotic genes function to specify the features that distinguish the segments. The proteins encoded by homeotic genes each contain the homeodomain, a 61-amino acid segment associated with DNA-binding activity, and they generally appear to be involved in control of gene expression at the level of transcription. The homeodomain has also been found in many other multicellular animals. In vertebrates, this evolutionary conservation extends to the organization of clustered homeodomain genes and to their sequentially ordered expression along the body axis. These similarities suggest that some of the mechanisms of regional specification, and perhaps even some aspects of segmentation and segmental differentiation, are conserved between insects and vertebrates.

Experiments from a number of laboratories suggest that the homeodomain itself is responsible for differences in functional specificity of *Drosophila* homeotic gene products. In an attempt to elucidate the mechanistic basis of functional specificity, Stephen Ekker, focusing initially upon the homeotic genes *Deformed* and *Ultrabithorax*, has shown that individual homeodomains bind DNA with similar but distinct base sequence preferences. These small differences in sequence recognition can be summed through cooperative binding to yield large overall differences in binding to multiple sites within a DNA region.

More recently the *Abdominal-B* homeodomain was found to exhibit a DNA base sequence preference distinct from *Deformed* and *Ultrabithorax*. In contrast, Donald Jackson showed that the base sequence preference of the *Antennapedia* homeodomain is similar to that of *Ultrabithorax*. Because *Antennapedia* and *Ultrabithorax* specify distinct segmental differentiation pathways, this result suggests that differences in functional specificity cannot be accounted for solely by intrinsic DNA sequence recognition properties. Other sources of functional difference are currently being investigated—in particular, the possibility of specific interactions with other proteins that act as DNA-binding partners or as links to other parts of the transcriptional apparatus.

A continuing effort in Dr. Beachy's laboratory is the identification and characterization of genes regulated by homeotic gene products. Dr. Chin Chiang has identified a gene encoding a homeodomain protein that is expressed in a specific subset of neuroblasts and in the anterior spiracle precursor cells in the first thoracic segment. Embryos in which *Ultrabithorax* function is deficient display a duplication of the spiracular precursor staining pattern in the second and third thoracic segments, and the other homeotic genes of the bithorax complex similarly appear to repress expression in the abdominal segments. Dr. Chiang is currently analyzing the phenotypes of mutations at the locus and the molecular basis for the negative regulation of this novel homeodomain gene by the homeotic genes of the bithorax complex.

Dr. Beachy is also Assistant Professor in the Department of Molecular Biology and Genetics at the Johns Hopkins University School of Medicine.

MOLECULAR STUDIES OF HUMAN GENETIC DISEASES

Arthur L. Beaudet, M.D., *Investigator*

Leukocyte and Endothelial Cell Adhesion Molecules

Inflammatory cell adhesion molecules (CAMs) on the surface of leukocytes and endothelial cells play a major role in inflammatory responses by mediating leukocyte-endothelial interactions, leukocyte-leukocyte interactions, leukocyte emigration from the vasculature, and other cellular processes. These molecules include leukocyte integrins (CD18, CD11a, CD11b, CD11c, VLA-4), members of the immunoglobulin superfamily (ICAM-1, ICAM-2, VCAM), and the selectins (P-, E-, and L-selectin). Dr. Beaudet's laboratory is examining the hypothesis that reduced levels of expression of the inflammatory CAMs will be correlated with reduced susceptibility to common human diseases that have an inflammatory component, such as atherosclerosis, inflammatory bowel disease, rheumatoid arthritis, insulin-dependent diabetes mellitus, and autoimmune diseases. Mutations in inflammatory CAMs are being introduced and tested in mice through gene targeting, to determine whether genetic variation in these loci might be an important variable in disease susceptibility. If the hypothesis is proved correct, pharmacologic methods to reduce expression or function of inflammatory CAMs should reduce or prevent disease processes that are strongly influenced by inflammatory CAM expression.

The CD18 gene encodes the β_2 subunit, which can combine with any of three different α subunits to form leukocyte integrins. CD18 deficiency in humans causes granulocytosis, impaired leukocyte emigration, serious susceptibility to bacterial infections, and early death in the severe form of the disorder. Human CD18 deficiency is an excellent candidate for somatic gene therapy using *ex vivo* infection of bone marrow cells. Toward this goal, human CD18 borne by a retrovirus vector has been used for expression in granulocytes in transplanted mice, for correction of the adhesion defect in deficient lymphoblasts, and for expression in deficient human bone marrow cultured *in vivo*. The relevance of more-modest variations in CD18 expression in common human diseases is unexplored.

To obtain a CD18 mutant mouse, the laboratory used an insertion vector for gene targeting. Homozygous mutant animals are healthy and fertile when raised in a specific pathogen-free (SPF) environment. Mutant animals have a moderate leukocytosis similar to that seen in mild forms of human CD18 deficiency. Immunostaining and fluorescence-activated cell sorting (FACS) analysis of mutant animals reveal a 70–90% reduction of surface expression of CD18 on lymphocytes and granulocytes.

Residual expression of CD18 was proved to be due to a cryptic promoter that lies in the plasmid backbone of the insertion construct just upstream of the exon containing the ATG initiator codon. This mutation provides a hypoinflammatory mouse model, and the effect of the mutation on various inflammatory diseases is being studied. There is a modest but significant delay in transplantation rejection in mutant animals used as recipients in neonatal heart transplants.

ICAM-1 is a transmembrane adhesion protein that, while relatively widely expressed, is particularly important on the endothelial surface, where it mediates leukocyte emigration through interaction with some of the β_2 integrins. No human deficient patients are known. A null mutation was introduced into the mouse ICAM-1 gene, using gene targeting with a replacement construct. Homozygous mutant mice are healthy and fertile in an SPF environment. Immunostaining of lung, where ICAM-1 is maximally expressed, reveals complete absence of expression in mutant animals, and immunostaining and FACS analysis of splenocytes from mutant animals similarly reveals a complete absence of expression. Examination of mutant animals in a chemical (thioglycollate) peritonitis model indicates reduced emigration of leukocytes to the peritoneal cavity—as reflected in elevated numbers of granulocytes in the peripheral blood and reduced granulocytes in the peritoneal fluid—although leukocyte emigration by ICAM-1–independent mechanisms is substantial.

To test the hypothesis that genetic variation in inflammatory CAMs might correlate with common disease susceptibilities, a series of DNA polymorphisms have been identified in some of these genes, with particular focus on ICAM-1 and the selectin gene cluster. All three known selectin genes are located within a 200-kb region on human chromosome 1. These polymorphisms, including a common amino acid polymorphism in ICAM-1, have been used for linkage analysis in humans and will be tested soon in appropriate populations to search for

evidence of disease correlations with coronary artery disease, inflammatory bowel disease, and insulin-dependent diabetes mellitus. Studies of disease association (linkage disequilibrium), sib-pair analysis, and linkage analysis will be performed.

Molecular Genetic Studies of Cystic Fibrosis

Cystic fibrosis (CF) is the commonest frequent lethal autosomal-recessive disease in Caucasians. Although the commonest mutation causing CF, a deletion of phenylalanine-508 (ΔF508), is present on 76% of Caucasian CF chromosomes, a large number of other mutations account for the remaining defects. For purposes of genetic counseling, prenatal diagnosis, carrier detection, and population screening, it is desirable to test DNA samples for as many different CF mutations as possible using efficient methods. Dr. Beaudet's laboratory has developed methods to test simultaneously for 22 different CF mutations, using robotics to perform most of the testing with allele-specific oligonucleotides in a combinatorial format. Analysis of 22 mutations detects 89.5% of CF carriers in a sample of more than 400 North American Caucasian CF chromosomes.

In addition, the ligase chain reaction (LCR) has been evaluated for feasibility of detecting any small mutation in a multiplex format. Using a competitive LCR method with two common oligonucleotides on one side of the mutation and four oligonucleotides (two for mutant and two for normal) on the opposite side, single-base mutations are detected routinely in this simple process.

Multiplex analysis of different mutations in the same reaction is also possible. Methods for simultaneous analysis of many single-base mutations will have general applicability for prereproductive screening and for disease-related testing in the population. Multiplex reproductive testing for diseases such as CF, Tay-Sachs disease, and Gaucher's disease is feasible, as is testing in the foreseeable future for a predisposition to emphysema, atherosclerosis, hemochromatosis, cancer risks, and other disorders.

In collaboration with Dr. Allan Bradley, gene targeting has also been used to introduce mutations into the CF gene of mice. Germline transmission of CF mutations has been obtained, and the mutant mice should be valuable for comparison with mice recently described by others, for studies of pathophysiology and for evaluation of pharmacologic trials and gene therapy.

The project to obtain CF mutant mice was supported by a grant from the Cystic Fibrosis Foundation.

Gene Cloning for Spinocerebellar Ataxia, Prader-Willi Syndrome, and Angelman Syndrome

A long-standing collaboration with Dr. Huda Zoghbi aimed at positional cloning for the spinocerebellar ataxia 1 gene continues. Highly polymorphic DNA markers that show no recombination with the disease locus have been obtained, and most of the candidate region is available in yeast artificial chromosome (YAC) clones. Candidate genes are being isolated from the region.

In another collaborative effort, the laboratory of Dr. David Ledbetter has isolated YAC clones for the majority of the candidate region for the Prader-Willi syndrome and Angelman syndrome genes, both of which lie within a 3- to 4-Mb region on human chromosome 15. These human mental retardation syndromes are of particular interest because there is strong evidence of genomic imprinting, with the Prader-Willi gene expressed only from the paternal chromosome and the Angelman gene expressed only from the maternal chromosome. Dr. Beaudet's laboratory is isolating candidate genes from the YAC clones with the intent to test for genomic imprinting, to search for mutations in Prader-Willi and Angelman patients, and ultimately to identify the disease genes. Four candidate cDNA clones isolated from this region are being analyzed.

Dr. Beaudet is also Professor in the Institute for Molecular Genetics and the Departments of Pediatrics and Cell Biology at Baylor College of Medicine.

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MOLECULAR GENETICS OF DIABETES MELLITUS

GRAEME I. BELL, PH.D., *Investigator*

Dr. Bell's laboratory is studying the molecular genetics of diabetes mellitus. The major interest is in the form that primarily affects aging individuals: non-insulin-dependent, or type 2, diabetes mellitus (NIDDM).

Genetics of Non-Insulin-Dependent Diabetes Mellitus

About 10% of adults in the United States are affected by NIDDM. The disorder is characterized by high blood glucose levels, which can result, if untreated, in cardiovascular disease, kidney failure, blindness, nerve damage, and early death. In contrast to patients with the less common insulin-dependent, or type 1, diabetes mellitus, who require daily insulin injections for survival, patients with NIDDM are able to produce insulin, but the amounts secreted by their pancreas are inappropriately low relative to their blood glucose levels.

Both genetic and nongenetic factors contribute to the development of NIDDM. However, the genetics of NIDDM is complex, and the mode of inheritance of NIDDM does not correspond to that of a simple dominant or recessive disorder. Moreover, there are likely to be a number of different genes whose mutation increases the risk of developing NIDDM. Identification of these genes is the goal of this laboratory. An understanding of the molecular basis of diabetes will lead to new and better forms of therapy and will facilitate the identification of presymptomatic genetically susceptible individuals, thereby allowing intervention programs to be initiated.

Because of the late onset of NIDDM, usually in middle age, the patients' parents are often not available for study, and the patients' children are too young to display symptoms of diabetes. Thus it is difficult to obtain large multigenerational pedigrees suitable for genetic studies. However, Dr. Stefan S. Fajans (University of Michigan) has described a slowly progressing form of diabetes occurring in some children, adolescents, and young adults that has a prominent familial character. This form, called maturity-onset diabetes of the young, or MODY, is highly penetrant and has an autosomal-dominant inheritance. It is therefore a good model for investigating the natural history of NIDDM. In all other respects, except for the age at onset, the clinical features of MODY are similar to those found in the more common late-onset form of NIDDM.

The largest and most thoroughly studied family with MODY is the R-W pedigree, a Michigan family of German origin. Their NIDDM is characterized by a delayed and subnormal insulin secretory response to glucose, suggesting that the primary defect responsible for the disease manifests itself in the insulin-secreting β cell of the pancreas. In collaboration with Dr. Fajans and Dr. Richard Spielman (University of Pennsylvania), Dr. Bell and his colleagues reported that the gene responsible for diabetes in the R-W family is on the long arm of chromosome 20. They have now narrowed its location to a region of ~10 million bp, and studies are under way to isolate it and identify the mutation that impairs its function. It will then be possible to study how genetic variation in this gene contributes to the development of the more common late-onset form(s) of NIDDM.

The past year saw the report of a second locus responsible for the development of MODY. Dr. Philippe Froguel (Centre d'Etude du Polymorphisme Humaine, Paris) reported close linkage of DNA polymorphisms in the glucokinase gene on chromosome 7 with early-onset NIDDM in a group of French families. In collaboration with Dr. Froguel, Dr. Bell and his colleagues have identified mutations in 18 of 32 French families with MODY. Dr. Bell and his colleagues have also identified mutations in the glucokinase gene in British, Swedish, Japanese, and African-American patients with NIDDM, indicating that they are not restricted to a particular ethnic or

racial group. To date, 20 different mutations have been identified in the glucokinase gene, and mutations in this gene represent the most common cause of NIDDM so far identified.

Glucokinase is expressed in the insulin-secreting β cells of the pancreas and in cells of the liver. It catalyzes the transfer of phosphate from ATP to glucose and plays an important role in regulating and integrating glucose metabolism in both tissues. In pancreatic β cells, glucokinase is believed to be the "glucose sensor" that modulates insulin secretion in response to changes in plasma glucose concentration. Dr. Bell has suggested that mutations in this gene may cause diabetes by a gene dosage mechanism. The presence of a mutation in one of two alleles of the glucokinase gene results in a decrease in the levels of glucokinase activity in pancreatic β cells. As a consequence, the threshold for glucose-stimulated insulin secretion is increased and higher levels of glucose are required to trigger secretion. Such decreased β-cell sensitivity to glucose can account for many of the clinical features of this type of NIDDM. The identification of diabetes-causing mutations in the glucokinase gene suggests that NIDDM may be primarily a disorder of glucose metabolism. This observation also suggests that other glycolytic enzymes, especially those that control rate-limiting steps in glucose metabolism, are candidates for contributing to the development of this clinically heterogeneous disorder.

Molecular Biology of Diabetes Mellitus

In addition to using genetic strategies for identifying diabetes susceptibility genes, Dr. Bell and his colleagues, with support from a grant from the National Institutes of Health, have continued to clone and characterize cDNAs/genes encoding proteins whose mutation or altered regulation may contribute to diabetes or its characteristic metabolic derangements. These clones can be used as molecular probes for genetic studies and for physiological studies examining the effects of diabetes on cellular processes. A major effort is under way in Dr. Bell's laboratory to isolate cDNAs encoding proteins that regulate insulin secretion by the pancreatic β cell. The group has isolated cDNAs encoding the α_1 subunit of the neuroendocrine-type voltage-dependent calcium channel and has shown that glucose represses this gene's expression, which suggests that its decreased expression may contribute, at least in part, to β-cell failure in NIDDM.

In addition, cDNAs encoding a third subtype of the inositol trisphosphate receptor, as well as a family of somatostatin receptors, have been isolated and characterized from pancreatic islet cDNA libraries. The inositol trisphosphate receptor functions in the mobilization of intracellular calcium, thereby facilitating insulin secretion. In contrast, the somatostatin receptors mediate somatostatin inhibition of insulin secretion. These studies represent the first steps toward deciphering the molecular mechanisms regulating insulin secretion.

Dr. Bell's multidisciplinary approach is providing a better understanding of the causes of diabetes mellitus. This will facilitate the development of new therapeutic approaches based on recognition that the disease has not one cause but many.

Other Projects

Dr. Bell, in collaboration with Rex Haydon (a graduate student in the Department of Anthropology, University of Chicago) and Dr. Jane Buikstra (University of Chicago), is isolating DNA from tissues of Chiribaya mummies (A.D. 1000–1300) from the west coast of South America. Their studies have shown that DNA of sufficient quality can be isolated from bone and mummified flesh from these archeological samples and that specific sequences can be amplified using the polymerase chain reaction. They propose to use molecular biology to reconstruct population structure and migrational events of these ancient peoples.

Dr. Bell is also Professor of Biochemistry and Molecular Biology and of Medicine at the University of Chicago.

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DIFFERENTIATION OF THE *DROSOPHILA* NERVOUS SYSTEM

Hugo J. Bellen, D.V.M., Ph.D., *Assistant Investigator*

Dr. Bellen and his colleagues study the molecular, cellular, and genetic mechanisms by which neural cells differentiate in the peripheral nervous system (PNS) during embryonic development.

RNA Processing May Require *couch-potato* in the PNS

In order to isolate PNS differentiation genes, whose expression may be controlled by proneural genes, about 4,400 enhancer detector strains were screened for β-galactosidase expression in the sensory motor cells (SMC) or PNS precursor cells. Fifteen enhancer detector strains that express *lacZ* in the SMC and map at cytological subdivision 90D were identified and genetically characterized. The gene adjacent to these insertions was shown to be essential and was named *couch potato* (*cpo*) because of severe hypoactive behavior of some adult mutant flies.

The *cpo* gene has now been cloned and characterized at the molecular level. It is complex and has two (or more) promoters and two transcripts. One of the transcripts is differentially spliced and forms two messages. Some of the messages, characterized by their cDNAs, are expressed in different cells of the PNS during development. Three different types of cDNAs were isolated, sequenced, and mapped. None of the three cDNAs contains a standard AUG initiation codon. Two of the three cDNAs encode putative proteins that are similar. Both proteins contain a carboxyl-terminal motif that is found in many RNA-binding proteins, termed the RNA-binding consensus. These domains are most homologous to similar domains of two proteins that are also expressed in the nervous system. One of these proteins has been implicated in a human sensory neuropathy. In collaboration with Dr. David Anderson (HHMI, California Institute of Technology), Dr. Bellen's laboratory has cloned and sequenced a mammalian *cpo* cDNA homologue. Both proteins contain very similar RNA-binding consensus sites, and it will soon be established whether these proteins are expressed in similar tissues in vertebrates.

Based on these and other genetic data, it is proposed that *cpo* expression is regulated by the proneural genes and that it carries out an essential differentiation function in the PNS. The Cpo protein probably modifies some of the RNA species that are produced in the cells in which it is expressed. These modifications may produce a number of proteins that are specific to the PNS. These proteins seem to be required mainly for a normal function of the PNS, as no obvious morphological defects have been observed in embryos that lack Cpo protein. (The project described above was supported by a grant from the Muscular Dystrophy Association.)

Neuromusculin, a Cell Adhesion Molecule

The *neuromusculin* (*nrm*) gene was identified in the same enhancer detector screen as *cpo*. It is also expressed in the SMC and the more differentiated cells of the PNS. However, in contrast to *cpo*, its expression in the embryonic cells of the PNS is transient. It is essentially absent in the PNS of embryos at stage 15 and older. At stages 15 and 16, prior to the formation of the neuromuscular junction, muscles start expressing *nrm*.

Sequencing of a number of *nrm* cDNAs showed that it encodes a molecule of the immunoglobulin-like (Ig-like) family with a signal peptide, nine Ig-like domains, a transmembrane domain, and a very short cytoplasmic domain. In addition, it contains 20 potential glycosylation sites and a single protease cleavage site in the membrane-proximal Ig-like domain. These observations suggest that Nrm is an extracellular protein that is membrane anchored and possibly secreted. Immunoblots with polyclonal antibodies against Nrm show that it is both associated with membranes and secreted by S2-expressing cells.

Expression of Nrm in S2 cells indicates that it is a weak homophilic cell adhesion molecule. However, mixing assays with Fasciclin II–expressing S2 cells and Nrm-expressing cells suggests that Nrm and Fasciclin II are homophilic and heterophilic cell adhesion molecules. Interestingly, Fasciclin II is abundantly expressed on the growth cones of many motoneurons prior to the formation of the neuromuscular junctions.

It is proposed that Nrm and Fasciclin II interact *in vivo* and that both proteins may play a role in setting up the neuromuscular junction. In addition, Nrm may function in growth cone guidance, as *nrm* is expressed in the PNS prior to the initiation of axonogenesis of motoneurons and sensory neurons. Since motoneurons express Fasciclin II and sensory neurons Nrm, and since both types of neurons fasciculate in the PNS, it is possible that their interactions play a role in peripheral axonogenesis.

Expression Pattern and Genetics of *Drosophila* Synaptotagmin, a Synaptic Vesicle–Specific Protein

The laboratory has recently embarked on a molecular and genetic analysis of *Drosophila synaptotagmin* (*syt*), in collaboration with Dr. Mark Perin of the Division of Neuroscience, Baylor College of Medicine. SYT is a synaptic vesicle–specific membrane protein that has been suggested to play a key role in synaptic vesicle docking and fusion. *In situ* hybridizations show that *syt* message is present in the cell bodies of many central nervous system (CNS) and all PNS neurons during neurite outgrowth, synapse formation, and in fully developed embryos. Immunocytochemical staining of embryos with antibodies to SYT show that it is only transiently localized to the cell body of the neurons and that most of it is transported rapidly along the axons during axonogenesis. In the CNS, SYT is mostly localized to two broad longitudinal tracts on the dorsal side of the ventral nerve cord and the brain and at the neuromuscular junctions in the periphery. These results, when compared with the data obtained from vertebrates, suggest that the molecular mechanisms governing the localization of the synaptic vesicle before and after synapse formation as well as the function of SYT may be conserved from invertebrate to vertebrate species.

Genetic analyses have allowed the mapping of the *syt* gene to cytological interval 23A4-23B2, which spans ~100 kb. Six homozygous lethal mutations that possibly fall into two complementation groups have been isolated and localized to this interval. Some allelic combinations of one of the complementation groups cause embryonic lethality, and these embryos show reduced muscle contractions. These and other preliminary data suggest that the mutations affect *syt*. It is hoped that they will allow *syt*'s function *in vivo* to be dissected. (This research is supported by the National Institutes of Health.)

Dr. Bellen is also Assistant Professor in the Institute for Molecular Genetics, the Division of Neuroscience, the Division of Developmental Biology, and the Department of Cell Biology at Baylor College of Medicine.

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HEMATOLYMPHOID PRECURSOR DEVELOPMENT

JOHN W. BELMONT, M.D., PH.D., *Assistant Investigator*

The production of mature blood cells depends on a tightly controlled developmental hierarchy of stem cells and committed progenitors. Dr. Belmont's laboratory is investigating the control of growth and differentiation of myeloerythroid and lymphoid precursor cells. The objective is to contribute to the understanding of the basic biology of these cells and to apply that information to the problems of immune deficiency diseases and human gene therapy.

Hematopoietic Stem Cells

A major goal is to be able to propagate stem cells in culture, since this would facilitate molecular analysis and have many medical applications. In order to clarify the role of the stromal cell interactions in the growth of stem cells in culture, Dr. Belmont's laboratory developed a novel strategy called mixed vector infection. A family of sister vectors was constructed by subcloning small random genomic DNAs into a *neo*-containing base vector (pN2). Each vector is easily distinguishable by polymerase chain reaction (PCR). The vectors were produced at high titer and then used in marrow transduction experiments. Clones of cells should have a characteristic pattern of vector integration. This system was used to examine growth in short-term culture of marrow-repopulating activity cells (MRA, cells that

seed the bone marrow space within two weeks of transplant) and the long-term repopulating cells (LTR, >6 months post-transplant).

At this time the only reliable method for analyzing the earliest hematopoietic precursors—the stem cells—is bone marrow transplantation. MRA and LTR stem cell clones (20/22) integrated single-vector types rather than representatives of the entire mixture in the culture. Southern analysis showed that multiple copies of that vector integrated into each precursor. These studies provide evidence for gene transfer and growth of the precursors in a restricted culture microenvironment.

A new culture system that apparently supports both lymphoid and myeloid precursor growth is now being evaluated, using this gene marking method. Since most efforts to transfer therapeutic genes into stem cells use retrovirus vectors, the finding that cell interactions are required for efficient vector transduction has important implications for human gene therapy methods.

Dr. Belmont's laboratory has also tested the potential for gene transfer in human hematopoietic stem cells. As a preliminary to critical *in vivo* tests, human long-term culture assays were exploited to measure the transduction of primitive human precursors—long-term culture-initiating cells (LTCIC). Differential vector marking was used to establish competitive long-term culture assays. These results highlighted the importance of stromal cell support for the LTCIC.

Transduction efficiencies of 60–70% were achieved under optimal conditions—i.e., cultivation of the target cells on preestablished stroma during the period of transduction. In contrast to the murine system, transduction efficiency could not be improved by stimulation with leukemia inhibitory factor (LIF), stem cell factor (SCF), interleukin-3 (IL-3), or IL-6. The procedures developed in these studies are very promising for use in clinical protocols and will be incorporated into studies being conducted by Dr. Albert Deisseroth (M.D. Anderson Cancer Center, Houston). (These experiments were funded in part by a grant from the National Institute of Allergy and Infectious Diseases, National Institutes of Health.)

Another way to approach the problem of stem cell growth is to examine the earliest embryologic specification of the precursor cells. Hematopoiesis first occurs in the yolk sac of developing embryos. In the mouse, blood islands develop from the extraembryonic mesoderm at day 7.5–8.0. This process can be modeled in culture, using embryonic stem (ES) cells. Under proper conditions ES cells form cystic embryoid bodies, up to 90% of which show active

hematopoiesis. Dr. Belmont's laboratory has recently detected cells with cell surface markers characteristic of primitive hematopoietic precursors in the embryoid bodies. The laboratory hopes to use this new culture system to identify genes important in lineage determination.

One additional approach is also being pursued—the identification of the growth factor receptors expressed by the stem cells. A systematic search for novel receptors and their ligands might lead to clarification of the molecular basis for growth control of these stem cells.

Using PCR, Dr. Belmont and his colleagues identified a new member of the receptor tyrosine kinase family by its expression in cells enriched for stem cell activity. Determination of its complete sequence is under way. Functional studies using the ES cell system should help to clarify its potential function in stem cells.

X-linked Agammaglobulinemia

Dr. Belmont's group is attempting to clone the gene mutated in Bruton's X-linked agammaglobulinemia (XLA), one of the commoner human primary immune deficiencies. Affected males fail to produce mature B lymphocytes as a result of a cell autonomous block in the transition of pre–B cells to more-mature forms. This condition provides a clinically important model for genetic control of lymphoid precursor differentiation.

XLA has been linked to Xq22. Flanking markers have placed it in an interval of ∼3.5 cM. An anonymous marker, DXS178, shows no recombination with the disease locus in more than 100 meioses. In collaboration with Dr. Mary Ellen Conley (St. Jude Children's Research Hospital, Memphis), the laboratory is using a combination of linkage analysis, yeast artificial chromosome (YAC) contig construction, and pulsed-field gel electrophoresis (PFGE) mapping to identify candidate genes. An incomplete YAC contig spanning ∼3 Mb around DXS178 has been constructed. One short tandem repeat (STR) polymorphism has been identified. This detects 11 alleles (heterozygosity ∼70%).

End probes from the YACs have been used in PFGE. At least three CpG islands have been identified in the area around DXS178. Lineage-specific differential methylation has been observed in two of the CpG islands, suggesting the possibility of regulated expression within the cell types of interest. Cloning of these putative genes is under way.

An important aid to linkage analysis and clinical genetic counseling is accurate carrier detection. Carrier females are clinically normal, but their B cells show exclusive use of the nonmutant X chromo-

some with respect to X inactivation. This presumably results from selection for the active normal X during B cell differentiation. Dr. Belmont's laboratory has developed a highly informative PCR-based assay for carrier detection, using differential methylation of *Hpa*II and *Hha*I sites near the polymorphic CAG repeat in the androgen receptor locus.

Dr. Belmont is also Assistant Professor in the Institute for Molecular Genetics, Department of Microbiology and Immunology, and Department of Pediatrics at Baylor College of Medicine and a member of the staff at Texas Children's Hospital and Ben Taub General Hospital, Houston.

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RETROVIRAL REPLICATION AND NEW METHODS FOR GENE MAPPING

Patrick O. Brown, M.D., Ph.D., *Assistant Investigator*

Retroviral Replication

Dr. Brown's laboratory is studying the mechanism by which a retrovirus delivers its genome into the nucleus of the infected cell and integrates the viral DNA molecule into a host cell chromosome. These steps are essential for retroviral replication. Retroviral integration provides a uniquely efficient means of inserting foreign DNA into mammalian chromosomes and thus can play a key role in genetic engineering and gene therapy. Moreover, since integration depends on virally encoded functions and has no known essential cellular counterpart, it provides a promising target for development of new antiviral agents. Two retroviruses are being studied in Dr. Brown's laboratory, the Moloney murine leukemia virus (MLV) and the human immunodeficiency virus (HIV).

Using an *Escherichia coli* expression system, postdoctoral fellows Drs. Samson Chow, Iris Dotan, and Karen Vincent and graduate students Viola Ellison and Brian Scottoline have prepared and purified milligram quantities of the wild-type HIV and MLV integrase proteins, as well as several mutant versions of HIV integrase. The integrase proteins have several activities that can be independently assayed, including sequence-specific endonucleolytic processing

of the viral DNA ends, cleavage of target DNA coupled to joining of viral and target DNA, and the reverse of the joining reaction, or "disintegration."

Dr. Vincent's analysis of the mutant forms of integrase has demonstrated that a highly conserved zinc finger–like motif near the amino terminus is not essential for catalysis or recognition of the terminal bases of the viral DNA, but may be important for recognizing features internal to the viral DNA end.

Dr. Chow's continued investigation of unorthodox substrates has clarified several aspects of substrate recognition and catalysis by integrase. First, when an especially favorable disintegration substrate is used, more than 10 catalytic events per integrase monomer are detected over a period of a few hours. Thus integrase can act as an enzyme. Second, integrase recognizes its DNA substrates in structures that deviate from the double-helical form. Indeed, substrates in which the viral DNA ends are not base-paired appear to be preferred, suggesting that integrase binding to normal viral DNA ends may involve separation of the two strands at the end. Third, by using disintegration substrates in which target DNA is truncated either on one side or the other of the junction with viral DNA, the integrase has been found to interact asymmetrically with the target DNA. Fourth, the active site of integrase is promiscuous in its selection of the hydroxyl group used as a nucleophile for the DNA transesterification reaction. A 2' hydroxyl can substitute for the 3' hydroxyl ordinarily involved in disintegration.

Analysis of the features that distinguish DNAs that can be used as analogues of viral DNA ends or as target DNAs in an *in vitro* integration reaction, carried out by Dr. Dotan and graduate student Timothy Heuer, has provided strong evidence for the existence of separate binding sites for viral DNA ends and target DNA. This observation is being pursued by 1) chemically crosslinking specific substrate DNAs to the enzyme, so as to identify the amino acids that make close contacts with key features of the substrates, 2) using selection from a degenerate sequence pool to identify optimal sequences for recognition by each binding site, and 3) screening for integrase mutants that have defects in recognition of either viral or target DNA substrates.

Work on HIV integrase, described above, was also supported by a grant from the National Institutes of Health.

Because the poor solubility of HIV integrase has frustrated attempts to obtain good crystals for x-ray crystallography, Dr. Brown and research assistant Elizabeth Kubalek have begun an effort to obtain two-dimensional crystals for structural analysis by electron diffraction. This approach is much less demanding of protein solubility than is ordinary three-dimensional crystallography. Moreover, it can allow ready examination of interactions between the protein and its ligands, since one face of the protein is freely accessible to solution. Essentially, the method requires that proteins be bound to the head groups of a lipid monolayer at an air-water interface. Once concentrated in this quasi–two-dimensional space, they are allowed to crystallize, a process that may require only minutes and typically no more than a few hours.

The first requirement for two-dimensional crystallography is that the protein of interest be capable of binding to the lipid monolayer. Therefore, to enhance the reliability and general applicability of the method, the team has synthesized novel lipids with nitrilotriacetic acid (nickel-chelating) head groups and glutathione head groups. These lipids should be useful as general reagents for binding histidine-tagged and glutathione-sulfotransferase (GST)-fusion proteins, respectively, to a monolayer at an air-water or other interface. Crystallography experiments will be an important focus of work in the next year. If initial efforts are successful, this method will be used not only for initial structure determination but also to examine interactions with DNA substrates and other ligands.

New Methods for Linkage Mapping in Complex Genomes

Dr. Brown and postdoctoral fellow Dr. Stanley Nelson have developed a new method for linkage mapping, termed genomic mismatch scanning. It should allow widespread application of highly efficient affected-relative-pair linkage mapping methods. The approach uses specialized enzymes that can recognize differences between two DNA sequences, to map all the regions of identity-by-descent between two relatives in a single procedure. In the past year the procedure has been tested using baker's yeast (*Saccharomyces cerevisiae*) as a model system, with highly successful results. Preliminary experiments with human DNA indicate that genomic mismatch scanning can be applied to linkage mapping in humans and other higher organisms. Continuing work on this project is now focusing on automating the procedure and adapting it to mapping human genes. The genomic mismatch scanning method will then be applied to very large human populations, with the goal of mapping genes governing disease susceptibility and diverse other complex and quantitative traits. The project described above was supported by a grant from the National Institutes of Health.

Dr. Brown is also Assistant Professor of Pediatrics and Biochemistry at the Stanford University School of Medicine.

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TARGETED MUTATIONS IN EMBRYONIC STEM CELLS: A MOLECULAR GENETIC DISSECTION OF DEVELOPMENT

Mario R. Capecchi, Ph.D., *Investigator*

Gene targeting in mouse embryo-derived stem cells can be used to introduce designed modifications into virtually any gene in the mouse. The advantage of this technology is that the investigator chooses both the gene and the mutation. Because of the limitations imposed by the generation time of mice and the size of the available colonies, it is not practical to rely on random mutagenesis to generate mice with specific mutations. Thus gene targeting provides the only available technology for achieving this goal.

Dr. Capecchi's laboratory is using gene targeting to determine the role of two sets of genes in mouse development. The first set is involved in localized developmental decisions through cell-cell signaling, and the second comprises members of a transcriptional complex involved in specifying positional value along the anterior-posterior axis of the mouse. In order to make better use of the gene targeting technology, parameters that influence the gene targeting frequency have also been analyzed.

Parameters That Influence Gene Targeting

The capacity to create predetermined genomic alterations is dependent upon the precision of the recombination reaction. Recently it has been suggested that "replacement vectors" mediate homologous recombination with low fidelity. In this particular study, fewer than 5% of the recombinants were the result of simple legitimate replacement. This novel lack of fidelity prompted scrutiny of potential differences in experimental protocols. The important variable turned out to be the length of the genome-homologous DNA sequence flanking the mutation. If the replacement vector was designed such that the desired mutation was flanked on both sides by several kilobases of DNA homologous to the target locus, replacement proceeded with high fidelity. If, on the other hand, the mutation was flanked on one side by <1 kb of target-homologous DNA, abortive recombination occurred.

A parameter that strongly influences the gene targeting frequency is the extent of homology between the targeting vector and the target locus. However, there were discrepancies in the literature on the importance of this parameter in determining the targeting frequency. Furthermore, there were claims that "replacement" vectors and "insertion" vectors targeted with very different frequencies. To clarify this issue, a systematic analysis of this parameter was undertaken, using both replacement and insertion vectors. The vectors behaved similarly with respect to their targeting efficiency and dependency on the extent of homology between the targeting vector and the target locus. Furthermore, when a targeting vector behaved anomalously, an unfavorable distribution of heterology between the vector and the target locus was shown to be the culprit.

The Mouse *box* Genes

Mice and humans contain homologues of many genes that control early embryonic development in *Drosophila*, including 38 genes that directly correspond to the *Drosophila* homeotic genes of the *Ultrabithorax* and *Antennapedia* complexes. The evolutionary conservation of these genes may reflect the inheritance of a whole transcriptional program for specifying positional information in the embryo. However, no known mutations of these genes have been identified in either mouse or humans that would allow direct assignment of function. For this reason, Dr. Capecchi is seeking to provide a genetic definition for the function of these genes by creating mice with null alleles in each gene. From such a systematic analysis, the laboratory hopes not only to reveal the phenotypes associated with inactivating any individual gene but also to define, through epistasis and molecular analysis of

combinations of mutations, how these genes interact to form a developmental network for specifying positional information.

To date, Dr. Capecchi and his colleagues have described the phenotype associated with inactivating two closely linked *hox* genes, *hox-1.5* and *hox-1.6*, and have initiated analysis of mice with six additional *hox* gene mutations. Interestingly, the phenotypes of *hox-1.5⁻/hox-1.5⁻* and *hox-1.6⁻/hox-1.6⁻* mice are completely different and nonoverlapping. Whereas *hox-1.5⁻* homozygotes exhibit defects in muscle, cartilage, and bone in a narrow region of the mouse, *hox-1.6⁻/hox-1.6⁻* mice exhibit primarily neuronal deficiencies in the hindbrain and cranial nerves. The *hox-1.5⁻/hox-1.5⁻* mice also exhibit defects of the heart, major heart arteries, and craniofacial structures. This complex set of deficiencies is remarkably similar to the pathology of humans with the congenital disorder DiGeorge's syndrome. Early death of *hox-1.6⁻/hox-1.6⁻* mice may be attributable to the hindbrain defects, whereas *hox-1.5⁻/hox-1.5⁻* mice appear to die from cardiovascular failure.

int-related Genes

In addition to disrupting genes participating in a transcriptional developmental program, the laboratory is also focusing on genes that mediate localized developmental decisions through cell-cell signaling. The *int*-related genes are excellent candidates for this second class. These proto-oncogenes were first identified in virally induced mammary carcinomas. Expression studies, however, suggested that their normal role is in development. The *int-1* gene, now designated *wnt-1*, is a homologue of the *Drosophila wingless* gene required to establish the posterior compartment of each parasegment. Clonal analysis of *wingless* mutations indicate that they are not cell autonomous, which is consistent with the *wingless* protein being made by one cell type, being secreted, and affecting the fate of neighboring cells.

Previously Dr. Capecchi and his colleagues showed that disruption of *wnt-1* in the mouse leads to a range of phenotypes from death at birth to survival with severe ataxia, as a result of a cerebellar deficiency. Histological analysis revealed severe abnormalities in the development of the midbrain and cerebellum. The range in phenotype was puzzling and provided concern as to whether the targeted mutation could be "leaky." More recently the laboratory identified a separate mutant allele of *wnt-1*, *swaying*, which could be shown by sequence analysis to be a null mutation. Its range of expressivity was the same as that of the original targeted mutation. Comparative analysis of the two mutants dem-

onstrated that the primary cause of the *wnt-1* null mutation is the lack of formation of the anterior lobe of the cerebellum. However, with variable expressivity, the effects of the mutation can spread anteriorly into the midbrain and posteriorly to encompass the posterior lobe of the cerebellum. It is hypothesized that a *wnt-1*–related gene can complement, with variable expressivity, the function of *wnt-1* in the midbrain and posterior lobe of the cerebellum.

Phenotypic analysis of *int-2⁻/int-2⁻* mice has just been completed. This proto-oncogene is a member of the fibroblast growth factor family of genes. The expression pattern of *int-2* is complex, but the defects associated with disruption of this gene are restricted to formation of the tail and inner ear. The defect in tail formation shows 100% penetrance, but the inner ear defect shows variable penetrance and variable expressivity. Both the vestibule and the cochlea are malformed. In some mice, however, one inner ear can be completely malformed, whereas the other appears normal. Classically, variability in expressivity was attributed to either leakiness of the mutation or variability in the genetic background of the affected individuals. Here the laboratory showed that the variability in expressivity in the *int-2⁻/int-2⁻* mice cannot be attributed to either of the above reasons. Rather, Dr. Capecchi and his colleagues postulate that the developmental program, among individuals with identical genetic backgrounds, must be interpreted differently from one individual to another.

Dr. Capecchi is also Professor of Human Genetics at the University of Utah School of Medicine and Professor of Biology at the University of Utah.

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MEDICAL GENETICS AND MODELS OF MUTATION AND THERAPY

C. Thomas Caskey, M.D., *Investigator*

Discovery of Fragile X Syndrome and Myotonic Dystrophy Mutations

Fragile X syndrome is an X-linked recessive mental retardation disorder. The discovery of the mutation responsible for fragile X syndrome, an expanding CCG triplet repeat, gave insight into the molecular basis of inherited diseases with the feature of "anticipation." Expansion of the CCG repeat occurred from generation to generation within families once the repeat number reached approximately 52. This triplet expansion led to decreased expression of the *FMR-1* (*fragile X mental retardation 1*) gene once the repeat number exceeded approximately 200. Recently, both FMR-1 mRNA and protein were found to be absent in fragile X syndrome. These discoveries collectively document a new mutational mechanism for humans (repeat sequence expansion) and identified the gene associated with the most common form of human mental retardation, fragile X syndrome.

Myotonic dystrophy, the most common severe myopathy of adults, is inherited in an autosomal dominant manner. "Anticipation" is a well-documented feature of myotonic dystrophy. Severe and frequently lethal infantile myotonia occurs in children of mothers (not fathers) with mild myotonic dystrophy. The myotonic gene was mapped to an ~1-Mb region of chromosome 19 by a consortium sponsored by the Muscular Dystrophy Association. Knowledge of the molecular basis of anticipation in fragile X syndrome made possible development of a simple scanning method for identification of an unstable triplet repeat within the myotonic dystrophy locus. This method identified both the myotonic dystrophy mutation (an AGC repeat) and the gene, myotonin protein kinase. Using simple diagnostic methods capable of detecting triplet expansion, families and physicians now have accurate DNA-based diagnostics for myotonic dystrophy. Recombinant bacterial methods allowed a simple means of synthesis and purification of myotonin protein kinase. DNA sequencing of skeletal and muscle forms indicates that different forms of the gene are expressed. Because protein kinases are involved in the regulation of target proteins, research efforts are now directed toward elucidation of the pathogenesis of myotonic dystrophy, including study of target protein(s) and effect on muscle function.

The scanning method developed for triplet repeats in the myotonic dystrophy gene is being used to initiate a search for other disease genes. Approxi-

mately 50 independent genes have been isolated that contain triplet repeats similar to those found in fragile X syndrome, myotonic dystrophy, and spinobulbar muscular atrophy. Such triplet sequences are highly mutable; these genes may therefore be associated with additional heritable diseases. The scanning methods in progress may rapidly identify these diseases.

Duchenne Muscular Dystrophy: Cause and Treatment

Rapid diagnostic methods for Duchenne muscular dystrophy (DMD) indicate that 75% of all mutations involve deletions, the endpoints of which are frequently found in intron 44 of the dystrophin gene. Approximately 38,000 base pairs of this mutational hotspot have been determined. A rapid polymerase chain reaction (PCR) method can be used to define the deletion site in individual patients to within 5,000 bp of this 2,400,000-bp gene. The sequence of the region has revealed three sequence repeats of unusual character, as well as a highly polymorphic simple repeat sequence. These regions are suspect for the hotspot deletion sites. Computer analysis and subsequent mRNA studies have identified an additional and unexpected gene within intron 44, oriented in the opposite direction to dystrophin.

Efforts to correct DMD defects in patients are directed toward gene transfer therapy using a minigene constructed from full-length mouse dystrophin cDNA. This minigene has succeeded in the correction of the *mdx* mouse deficiency of dystrophin when regulated by a muscle-specific promoter and injected into the single-cell mouse embryo. Such a correction proves the utility of the minigene, but this strategy is not useful in humans. Viral delivery methods are under study for correction of the human disease. Retroviral and adenoviral vectors cannot accommodate the 11,000-bp minigene, and thus truncated versions of the full-length cDNA are being constructed. A 3,000-bp section of the spectrin repeat was removed in one construction; a 5,000-bp region was removed in a second. Furthermore, 2,500 bp were removed from the 3'-untranslated region of both minigenes. Each of the four truncated minigenes is being inserted into both adenoviral and retroviral vectors for study of dystrophin expression. In preparation for human studies, primary muscle cells have been isolated from eight DMD patients. This project was supported by a grant from the Muscular Dystrophy Association.

Gene Therapy for Adenosine Deaminase Deficiency

The ability to transfect pluripotent stem cells is paramount to the success of gene therapy by bone marrow transplant, because stem cells have the potential for both differentiation into all hematopoietic lineages and self-renewal. Recent efforts have focused on transfection of the stem cell–containing population bearing the CD34 glycoprotein on the cell surface. Colonies from transfected CD34$^+$ cells that were maintained in long-term culture for more than five weeks demonstrated 40–60% infection efficiency of the adenosine deaminase (ADA) gene based on proviral integration. ADA enzyme expression in transfected cells from ADA-deficient bone marrow approached levels seen in normal subjects. Current efforts are concentrated on the design, optimization, and approval of a clinical trial utilizing autologous stromal support for retroviral supernatant infection of CD34$^+$ bone marrow progenitor cells. Since the stem cell–containing population is also thought to be present in peripheral blood, studies have commenced on the potential use of CD34$^+$ cells selected from mononuclear cells obtained during stem cell pheresis for transfection and long-term expression of the transfected ADA gene. This project was supported by a grant from the National Institutes of Health.

Generation of Animal Models of Human Disease

Attempts to construct an ADA-deficient mouse are in progress using embryonal stem cell technology. This approach will replace normal regions of the gene by recombination with a mutation vector. Such technology was previously used to create a uricase-deficient mouse. The availability of these mouse models will permit both the study of ADA- and hypoxanthine guanine phosphoribosyltransferase (HPRT)-related immune deficiency and Lesch-Nyhan syndrome.

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RNA CATALYSIS AND THE STRUCTURE OF CHROMOSOME ENDS

THOMAS R. CECH, PH.D., *Investigator*

One goal of the research in Dr. Cech's laboratory is to understand how RNA molecules catalyze biochemical reactions. RNA-mediated catalysis is involved in many RNA-processing reactions, which are essential steps in gene expression, and RNA catalysis appears to be central to protein synthesis on ribosomes. Dr. Cech's research group continues to use the self-splicing intron from the *Tetrahymena thermophila* rRNA precursor as a model system for studying structure-function relationships in RNA catalysis, although other ribozymes (catalytic RNA molecules) are also being investigated. In the very different area of chromosome structure, Dr. Cech and his colleagues are studying DNA-protein interactions at telomeres, the natural ends of linear chromosomes.

Tertiary Structure Around the Guanosine-binding Site of the *Tetrahymena* Ribozyme

A cleavage reagent designed to bind to the active site of the ribozyme was synthesized by derivatizing guanosine, normally a substrate in the RNA-catalyzed reaction, with a metal chelator. This reagent, when complexed with iron(II), cleaved the ribozyme in five regions. One region at A207 is far from the guanosine-binding site in the primary and secondary structures, and therefore provides a constraint for the higher-order folding of the ribozyme. This cleavage site provides physical evidence for a major feature of the three-dimensional structure model of Drs. François Michel and Eric Westhof. This sort of targeting of a reactive species to a spe-

cific site is expected to be generally useful for determining proximity within folded RNA molecules or ribonucleoprotein complexes.

A New Class of Chemical Reactions Catalyzed by RNA

All the known classes of ribozymes catalyze reactions at phosphorous centers, but no reaction at a carbon center had been demonstrated. The active site of the *Tetrahymena* ribozyme was engineered to bind an oligonucleotide derived from the 3' end of *N*-formyl-methionyl-tRNA^{fMet}. This ribozyme was found to have a modest level of aminoacyl esterase activity, accelerating the hydrolysis of the fMet by as much as 15-fold beyond the uncatalyzed rate. Study of the ribozyme sequence requirements and magnesium ion requirement for the reaction indicated that it takes place in the same active site responsible for the normal endonuclease reaction. The ability of RNA to catalyze reactions with amino acid substrates expands knowledge of the catalytic versatility of RNA and suggests that the original aminoacyl tRNA synthetase could have been an RNA molecule.

Crystallization of Ribozymes for X-ray Diffraction Analysis

Although RNA structure and transition state interactions can be inferred from biochemical experiments, physical methods such as x-ray diffraction and nuclear magnetic resonance (NMR) provide the only sure paths to obtain atomic-resolution structures. In collaboration with Dr. Craig Kundrot (University of Colorado at Boulder), a systematic approach has been developed for crystallization of a number of ribozymes and structural domains thereof. A number of these RNAs have crystallized in a reproducible manner. Most current crystals are too small to attempt diffraction, but a 160-nucleotide independent folding domain of the *Tetrahymena* ribozyme has yielded crystals larger than 0.1 mm in each dimension. Diffraction studies are in progress. (This work has also been supported by the W. M. Keck Foundation.)

Engineering Ribozymes for Better Activity and Specificity

The RNA-substrate–binding site of the *Tetrahymena* ribozyme is connected to the catalytic core by the joining region J1/2. Small deletions in J1/2 were found to enhance dramatically the turnover number and sequence specificity of the ribozyme in the endonuclease reaction. Ironically, the explanation for these improved properties was found to lie in the decreased affinity of the mutant ribozyme for its RNA substrate, rather than improvement of the chemical cleavage step.

Ribozyme Tertiary Interactions Involving the RNA Backbone

In the endonuclease reaction of the *Tetrahymena* ribozyme, the helix P1, which contains the RNA cleavage site, must be juxtaposed to the guanosine-binding site. In a search for a nucleotide in the catalytic core that contributes to the stability of this arrangement, a conserved adenine (A302) was identified. Binding and activity studies with chimeric oligonucleotides and mutant ribozymes led to a model in which A302 accepts an H-bond from the 2'-OH group three nucleotides from the cleavage site in P1. The model was further tested and supported by methylation footprinting experiments. Such base-backbone tertiary interactions may be generally important in RNA folding.

Human RNA- and DNA-binding Proteins Specific for the Telomere Repeat Sequence

In previous work of Dr. Cech's laboratory, a proteinaceous activity was identified in HeLa cell nuclear extracts that binds to single-stranded repeats of TTAGGG, the human telomere DNA sequence. More-recent studies indicated that the proteins also bind to the corresponding RNA sequence UUAGGG, with higher affinity than to DNA. Purification based on sequence-specific binding gave a small group of proteins in the 37- to 41-kDa range. Amino acid sequence analysis (Dr. Clive Slaughter, HHMI, University of Texas Southwestern Medical Center at Dallas) identified peptides that matched known hnRNP E protein sequences, as well as a novel peptide containing the RNP 1 consensus sequence found in many single-stranded DNA- and RNA-binding proteins. Immunological reactivity (in collaboration with Dr. Gideon Dreyfuss, HHMI, University of Pennsylvania) identified the proteins as being related to hnRNP D and E proteins but, interestingly, not the D and E proteins found in hnRNP complexes. The functional relationship of these proteins to nuclear RNA and to telomeric DNA remains to be determined.

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GENOMIC SEQUENCE COMPARISONS

George M. Church, Ph.D., *Assistant Investigator*

Several laboratories are sequencing small genomes (1–100 Mbp) from each of the three phylogenetic kingdoms. Comparisons of these sequences will define consensus sequences for most classes of protein domains, evolutionary conservation, and change. Up to 20-fold higher substitution rates in nonconserved compared with coding nucleotides allow the discrimination of random open reading frames (ORFs) from those encoding proteins that confer a selective edge. The exceptionally extensive genetic maps, biochemical pathways, and regulatory landmarks for these organisms reveal physiological relationships and clues to functions for new sequence elements. The genome closest to completion is *Escherichia coli*, with more than 40% of its 4.7 Mbp completed by the work of 2,000 scientists. Dr. Church's laboratory is focusing on developing technology of general use in genome-sequencing projects, with emphasis on ways to improve accuracy and biological interpretability.

DNA Sequencing and Automation

Multiplexing is a general method of mixing and separating informational packets. In multiplex sequencing, pools of up to 40 strategically tagged DNA samples flow as if one throughout most of the protocol steps. Direct-transfer electrophoresis modified for multiplex and duplex sequencing yields runs with error rates <0.06% for the first 400 bases and <0.6% for the first 800 bases. The new chemiluminescent substrate CSPD yields three times faster exposures—in the range of 10 to 60 min. An automated PC-controlled drum device now handles the hybridization, washing, and substrate addition steps.

Dr. Church's laboratory has recently integrated direct-transfer electrophoresis, automated multiplex hybridizations, and automated film reading to sequence three *E. coli* cosmids. Sequence patterns for two cosmids were detected using chemiluminescence with oligonucleotide probes directly conjugated to alkaline phosphatase. Primers for the directed walking and dideoxy sequence confirmation steps contain 15 base tags for detection. For the cosmids, 20 gels resulted in 9,216 sequences on film. The programs REPLICA and GTAC automatically read and assemble the data from films. Another program automatically finds and graphically annotates ORFs, including matches to database sequences.

Along with many other features in the cosmids, this effort turned up new potential operon(s) covering 13 consecutive long ORFs without homologues in any databases. This is by far the longest operon found in *E. coli* or *Salmonella*.

To increase the number of oligonucleotides synthesized in parallel and decrease the cost of synthesis, solid-phase DNA synthesis has been multiplexed. Synthesis in this new system occurs on the surface of pins that dip into appropriate common troughs containing reagent, including modified monomers. A solenoid array controls these pins set on a 96-well 9-mm spacing, and a stepping motor positions the troughs. The synthetic scale has been miniaturized down ~1,000-fold to the 100-pmol range. The products pass quality control tests, including priming dideoxy-sequencing reactions, polymerase chain reactions, kinase labeling, and gel electrophoresis.

Computational Methods

The large number of sequence films produced by multiplexing are digitized with film scanners, allowing linkage of automatic base assignments and high-resolution images in a database. The computer program REPLICA uses internal standards from multiplexing to establish lane alignment, lane-specific reaction rules, and deconvolution models responsive to variations in the interband distance at different points on the sequencing runs. Images with overlapping data can be viewed side by side to facilitate decision making and automatic multisequence alignments. Image, contig, and sequence assignments from a multigigabyte database now correlate and display in <4 s. A sequence assembly system for ordered and shotgun data called GTAC has been improved and benchmark tested, using data from 12 cosmid projects with >300 kb of raw data each and from various simulations. The run times are nearly linear ($N^{1.1}$) with increasing project size (N). A sample data set of 2 million bases of raw DNA sequence with 2% error assembles in 9 h on a desktop computer (DEC VS 3100).

Linking DNA and Protein Databases

To test predictions of protein structure and abundance based on genomic sequence and consensus motifs, proteins are sequenced directly from two-dimensional gel spots. This also allows the two-dimensional databases on gene expression levels for various cell types and cell environments to be correlated to candidate DNA, RNA, and protein regulatory motifs. About 400 *E. coli* amino-terminal sequences have been obtained and compared with all DNA database sequences translated in all six frames. About 20% are unknown or match uncharacterized ORFs. Results on total cell extracts and various subcellular fractions indicate that proteins present anywhere from 100,000 copies to <1 copy per cell can be analyzed in this system.

Measurements of Genomes Using Single Ion Channels

Since patch-clamp conductance can measure subtle changes in single protein-ion channels in biological lipid bilayers, the possibility of studying DNA passing through or past such channels has been explored. This may not only provide a view of interesting genome movements—such as viral DNA injection, conjugative single-stranded DNA transfer, and polymerase function—but may ultimately lead to ways to read long DNA sequences rapidly. The initial focus will be on λ DNA injection into LamB protein pores.

Pathological Genome Sequences

Dr. Church's laboratory has begun surveying human DNA polymorphisms in G protein–coupled receptor genes associated with behavioral abnormalities and differential drug responsiveness. In conjunction with the genome group at Collaborative Research Inc., the multiplex system is being applied to the analysis of genomes of *Mycobacteria* species involved in tuberculosis and leprosy. The differences between the *E. coli* standard strain (K12 EMG2) and related pathogenic enterics are being explored by subtractive methods, in collaboration with laboratories at Massachusetts General Hospital.

Dr. Church is also Assistant Professor of Genetics at Harvard Medical School.

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MOLECULAR GENETICS OF LIMB DEVELOPMENT IN *DROSOPHILA*

STEPHEN M. COHEN, PH.D., *Assistant Investigator*

The organization of a complex three-dimensional body plan in a developing embryo requires that individual cells acquire distinct developmental fates. Cells become progressively distinguished from one another by alterations in their pattern of gene expression. These changes must be integrated so that neighboring cells adopt compatible developmental programs. Intercellular signaling systems are thought to play a fundamental role in the allocation of groups of cells into functional developmental units. Research in Dr. Cohen's laboratory is focused on one such system, the specification of the limb primordia in the *Drosophila* embryo. *Drosophila* provides an amenable system in which to study the genetic signals required to allocate a population of founder cells as the progenitors of limbs.

Origin of the Embryonic Limbs

Expression of the *Distal-less* gene provides the first indication that the limb developmental field has been established in the embryo. The *Distal-less* gene is expressed in the limb primordia prior to any overt morphological sign of limb development. This observation supports the suggestion that *Distal-less* expression might be an early cue that initiates commitment of embryonic cells to limb development. Genetic evidence indicates that the product of *Distal-less* is required for the specification of these embryonic cells as limb precursors. Embryos that lack *Distal-less* gene activity fail to develop any limb structures. *Distal-less* encodes a protein with features expected of a transcription factor. Consequently the gene is expected to exert its function by regulating additional subordinate target genes. The *Distal-less* protein is nuclear, binds to DNA in a sequence-specific manner, and activates transcription in a heterologous system (yeast).

In the *Drosophila* embryo, *Distal-less* is required at two stages. First, it must specify development of the larval limbs and, subsequently, the progenitor cells of the adult limbs. The discrete requirements for *Distal-less* activity in these processes have been shown to be controlled independently by different cis-regulatory elements that direct *Distal-less* expression in appropriate cells at the appropriate time.

Spatial and Temporal Control of *Distal-less* Expression

The leg primordia arise as distinct clusters of cells at well-defined positions in the embryo. It is of obvious importance that the positioning of the limbs be carefully regulated with respect to the rest of the body pattern. Both the establishment and positioning of the limb primordia were shown to depend on spatial cues provided by the segmentation genes. In particular, the *wingless* gene was implicated as the source of a positional cue locating the limb primordia with respect to the anterior-posterior axis of the embryonic segments.

An additional source of information is required to locate the limb primordia with respect to dorsal-ventral position in the segment. Preliminary studies implicate the *decapentaplegic* gene, which is involved in the organization of dorsal-ventral pattern in the embryo, where it is expressed on the flank in longitudinal stripes of cells. These stripes intersect the stripes of *wingless* gene expression at the precise location where the limb primordia develop.

The embryo locates its limbs at the intersections of lines of positional information drawn by the segmentation and dorsal-ventral genes. Interestingly, both *wingless* and *decapentaplegic* encode secreted signaling molecules. It seems probable that cells receiving both signals may be instructed to become limb cells. An early acting cis-regulatory enhancer element responsible for directing *Distal-less* expression in response to these intercellular inductive signals has been defined.

Repression of Abdominal Limb Development

Insects evolved from more primitive arthropods in which legs develop in all body segments. Limb development in the abdominal segments of insects is suppressed through the activity of the homeotic genes of the Bithorax complex. These genes have been shown to act by repressing expression of the *Distal-less* gene in the abdominal segments. The Bithorax complex genes encode homeodomain transcriptional regulatory proteins that have been shown to function as repressors. These proteins bind to the cis-regulatory enhancer element that controls *Distal-less* in the early embryo and thereby repress *Distal-less* expression in the abdominal segments.

The binding sites in the *Distal-less* enhancer to which these proteins bind *in vitro* are essential for the activity of the repressor element *in vivo*. Enhancers in which the sites have been specifically mutated to preclude binding are unable to confer repression by the Bithorax genes in the embryo. Therefore *Distal-less* acts as a direct downstream

target gene through which the homeotic genes control segment identity.

Downstream Target Genes through Which *Distal-less* Exerts Its Activity

To understand the role of *Distal-less* in organizing spatial pattern, it is important to identify the genes through which it acts. A number of prospective candidate target genes have been identified by virtue of their patterns of expression in the embryo. These genes are under genetic control of *Distal-less* in the embryonic limb primordia. The question of whether they are direct targets for transcriptional regulation by *Distal-less* is being addressed, and their contribution to limb development assessed.

Comparison of Pattern Formation in Wing and Leg

Distal-less is expressed in the presumptive adult limb structures throughout their development, but is not expressed in the surrounding body wall progenitor cells. In the absence of *Distal-less* function, body wall structures develop normally, but limb structures are entirely absent. *Distal-less* function is not required in the dorsal appendages to support the development of wing (as opposed to body wall). A number of mutants have been described that lack wings, including *apterous*. Null mutants show a complete deletion of wing structures. The *apterous* gene has been cloned and characterized. It encodes a predicted transcription factor of the LIM family, which is expressed in the dorsal surfaces of the wing and the body wall. Therefore, although the phenotypic consequences of removing *apterous* from the wing show some similarity to the effects of removing *Distal-less* from the leg, the genes' functions are not directly comparable in molecular terms.

Dr. Cohen is also Assistant Professor of Cell Biology and of Molecular Genetics at Baylor College of Medicine.

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MOLECULAR GENETICS OF HUMAN DISEASE

Francis S. Collins, M.D., Ph.D., *Investigator*

Almost all diseases have a genetic component, but for many the underlying biological defect has remained elusive. A powerful method for identifying such genes, known as positional cloning, has recently emerged. It utilizes the inheritance pattern of the disease within families to pinpoint the gene on a specific chromosome, followed by intensive searching of that region to identify a gene that produces abnormalities in affected individuals. The Collins laboratory is currently pursuing four disease genes whose molecular basis has been or will be uncovered by this strategy.

Familial Early-Onset Breast Cancer (BRCA1)

A positive family history increases an individual's risk for developing breast cancer, and in some remarkable families with many affected women the inheritance of this disease appears to be a pure autosomal dominant. This early-onset breast cancer gene was mapped to chromosome 17 in 1990 by Dr. Mary-Claire King and her colleagues (University of California, Berkeley). The Collins laboratory, in collaboration with Dr. King, has initiated a broad series of efforts to identify the gene and find the mutations within it that carry such a high risk of the development of cancer. In addition to breast cancer, it is clear that the same gene is involved in many cases of ovarian cancer.

Over the past year, in close collaboration with Dr. Barbara Weber (Division of Hematology and Oncology, University of Michigan), a large number of affected families have been identified, DNA samples have been obtained, and linkage analysis has been carried out. Several of these families do indeed appear to carry the chromosome 17 gene, and in one of them a crucial recombinant event has allowed narrowing of the responsible interval to about half its previous size. An intense effort is now under way to clone this entire region, using a variety of novel technologies, including chromosome microdissection, radiation hybrids, fluorescent *in situ* hybridization, and yeast artificial chromosome (YAC) clon-

ing. The majority of the interval is now cloned, and a search for candidate genes has begun. The identification of the responsible gene, which is estimated to be present in approximately 1 in 200 women, would allow widespread screening in order to identify women at high risk. Given that intensive screening is often likely to be lifesaving, such a test could find wide application and be a major step forward in preventive medicine.

Huntington's Disease

In collaboration with six other laboratories in the United States and the United Kingdom, the Collins laboratory is pursuing the gene for Huntington's disease, which has been narrowed down to an interval of ~2.3 million base pairs on the short arm of chromosome 4. A complete set of YACs that cover this interval has been constructed, and those cloned fragments are now being used as the substrate for identifying candidate genes.

This has involved the development of new technologies capable of scanning such large intervals for coding regions. One such technique in which cDNAs are trapped against genomic DNA using a magnetic capture method appears to have broad promise for the rapid identification of a large number of genes in one experiment and has yielded several attractive candidates. It is estimated that approximately 100 genes will be contained within this large interval, and the proof of the correctness of any one candidate will depend upon the identification of a single mutation, which may be as subtle as a single nucleotide change. A large-scale sequencing effort is also being mounted in order to speed this activity.

Cystic Fibrosis

The cystic fibrosis gene, identified by the Collins laboratory in collaboration with investigators at the Hospital for Sick Children in Toronto in 1989, continues to produce a series of interesting observations that are further clarifying the basic molecular biology of the disease and beginning to suggest new modes of therapy. In collaboration with Dr. David Dawson, the Collins laboratory has been successful in expressing wild-type CFTR (cystic fibrosis transmembrane regulator) and various naturally occurring cystic fibrosis mutants in *Xenopus* oocytes (frog eggs). The normal CFTR RNA produces a dramatic chloride flux after cyclic AMP activation, providing an excellent model for looking at structure/function characteristics of CFTR.

Surprisingly, the common ΔF508 CFTR mutation, which had previously been assumed to inactivate the gene completely, turns out to have partial activity in this assay. Using drugs that maximally elevate cyclic AMP, the ΔF508 protein can be induced to approximately wild-type levels of current. Although much remains to be determined regarding the basis of this effect, the observation suggests the possibility of a new form of drug therapy that could activate the mutant in the airways of affected individuals. Extensive investigations are also under way to identify the regulation of gene expression of CFTR and to understand the mechanism by which the protein is activated during phosphorylation.

Neurofibromatosis

The gene for type 1 neurofibromatosis (NF1) was identified in the Collins laboratory in 1990, and progress continues in defining the molecular biology of this common dominant disease. A variety of antisera, raised against synthetic peptides and fusion proteins for the NF1 gene product, have been used to identify the protein by Western blotting, immunoprecipitation, and immunofluorescence. The protein is expressed in all tissues examined, but has highest levels in the central nervous system. It has homology to the GTPase-activating protein (GAP), which interacts with the oncogene product ras, and this relationship is being dissected by carrying out mutagenesis experiments on neurofibromin, the protein product of the NF1 gene. Immunofluorescence experiments have demonstrated the unexpected finding that the NF1 protein appears to be associated with microtubules. This connection between ras-mediated signal transduction and the cytoskeleton represents a totally unexpected finding, and one that may be fundamental to the regulation of cell division.

In addition, the full-length cDNA for NF1 has been cloned and the entire sequence determined, and the genomic sequence has been obtained in a series of YACs that cover the entire gene.

A recent surprising observation is the discovery of frequent mutations in this gene in patients with sporadic malignant melanoma, the most common form of fatal skin cancer. This further broadens the role of NF1 in human disease.

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MOLECULAR ANALYSIS OF RNA POLYMERASE II

JEFFRY L. CORDEN, PH.D., *Associate Investigator*

Research in Dr. Corden's laboratory continues to focus on the unusual repeated sequence at the carboxyl terminus of the largest subunit of RNA polymerase II. This carboxyl-terminal domain (CTD) consists of tandem repeats of a seven–amino acid consensus sequence, TyrSerProThrSerProSer. This sequence is repeated from 17 to 52 times in different organisms. The CTD plays an essential role in the transcription process, but the mechanism by which it acts is unknown. Studies ongoing in Dr. Corden's laboratory are designed to elucidate the structure and function of this domain, using yeast and mouse as model systems.

The unusual sequence of the CTD suggests that it adopts a repetitive turn structure. Previous work from Dr. Corden's laboratory demonstrated that phosphorylation of serine residues in positions two and five of each repeat results in a conformational change that stiffens or unfolds the CTD. To address the molecular basis of this change, the structure of the phosphorylated and unphosphorylated CTDs are being compared. Nuclear magnetic resonance (NMR) spectra of model CTD peptides suggest the presence of two separate β-turn structures in each repeat of the unphosphorylated CTD. How these turns are linked in the full-length CTD is now under investigation.

In addition, the role of serine residues in maintaining the structure of the CTD is being addressed by studies of model peptides in which the phosphorylatable serines have been changed to alanine or glutamate. These CTD repeat variants are identical to the mutant CTDs that have been genetically tested in yeast cells.

To investigate the function of the CTD, the laboratory has initiated a comprehensive genetic analysis of the yeast CTD. The purposes of this analysis are 1) to determine which CTD sequences are essential for cell viability, 2) to determine the basis of the slow-growth phenotype of some CTD mutations, and 3) to isolate suppressors of CTD mutants and thereby identify molecules that interact with this domain.

As a first step in this analysis, a strategy to make point mutations in every repeat of the CTD has been developed. This was achieved by reconstructing the CTD coding sequence from synthetic oligonucleotides coding for either wild-type or mutant repeats. These synthetic CTDs are then cloned into a specially constructed polymerase expression vector. Several types of CTDs have been constructed and analyzed. First, deletion studies confirmed the results of previous experiments showing that CTDs with more than 11 of the normal 26 repeats behave identically to wild type. The minimum viable CTD is eight repeats long, and this strain exhibits a severe slow-growth phenotype.

The expression of some inducible genes has been shown to be reduced in short CTD strains. This cannot, however, explain the slow growth seen in rich media. In Dr. Corden's laboratory, clones of several genes underexpressed in the short CTD strain were identified, using differential screening. All five differentially reduced genes identified in the screen are glycolytic enzymes.

Based on this initial observation, the expression of other glycolytic enzymes was tested and shown to be reduced in a short CTD strain. The enzymes of the glycolytic pathway make up about 60% of the cytosolic protein in yeast and therefore represent a large percentage of transcription initiation events in the cell. However, the three- to fivefold reduction seen in the short CTD strain is not simply a reduction in the most highly transcribed genes, as several highly expressed genes that do not encode glycolytic enzymes are not reduced. Rather, the CTD is probably involved somehow in the specific expression of the genes encoding glycolytic enzymes. These genes have several transcription factors in common, and current studies are aimed at determining the role of the CTD in their action.

Another important finding is that RNA polymerase II that lacks the CTD has a dominant negative phenotype. Photoaffinity labeling experiments indicate that this enzyme is incapable of transcribing *in vivo*. Its ability to interfere with transcription by the wild-type enzyme might, therefore, be due to competition at the level of formation of the initiation complex. If the CTD-less enzyme can bind the promoter but not initiate transcription, then it may interfere with binding and initiation of the wild-type enzyme. Current *in vitro* studies are designed to test this interference hypothesis.

Mutations that lengthen the CTD have just as serious a consequence as the short CTD mutants. In addition to growing slower than their wild-type counterparts, the long-tail subunit is aberrantly phosphorylated in yeast. One suppressor of the long-tail mutant maps to the *GRR1* gene, which was previously shown to be involved in protein dephosphorylation. Whether this protein plays a role in transcriptional regulation is now under investigation.

Actively-transcribing RNA polymerase II is known to be highly phosphorylated on the CTD, and this modification is thought to take place during the initiation reaction. Dr. Corden's laboratory previously identified several protein kinases that are able to phosphorylate the CTD at serines in positions two and five. These residues have been mutated to alanine or glutamate, and the effect of these changes has been shown to be lethal. This observation is consistent with a requirement for phosphorylation of these residues *in vivo*.

Genetic suppression of lethal CTD mutations is being developed as a method for identifying proteins that interact with the CTD. Suppressors have been isolated that allow the growth of mutations in the CTD that would otherwise be lethal. A collection of 60 different strains containing suppressors of four different phosphorylation-site mutations are currently being characterized. At least one of these suppressors seems not to support growth in the presence of wild-type CTD, indicating a potential change in specificity of an enzyme or other protein that interacts with the CTD.

Previous studies in Dr. Corden's laboratory indicated that the CTD of mammalian species is highly conserved through evolution. This observation was unexpected in light of genetic studies showing that nearly half of the mouse CTD can be deleted without impairing the growth of cells in culture. Dr. Corden's laboratory is currently testing the hypothesis that the CTD plays a role in transcription during early development.

The laboratory has also continued a collaboration with Drs. Terry Brown and Claude Migeon of the Johns Hopkins University School of Medicine on the function of the human androgen receptor (AR). Dr. Corden's laboratory is currently investigating the mechanism by which the AR interacts with the basal transcription machinery to activate transcription of androgen-responsive genes.

Dr. Corden is also Associate Professor of Molecular Biology and Genetics at the Johns Hopkins University School of Medicine.

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REGULATION OF GENE EXPRESSION IN HIV-1 AND OTHER COMPLEX RETROVIRUSES

Bryan R. Cullen, Ph.D., *Associate Investigator*

The focus of research in Dr. Cullen's laboratory continues to be on the regulation of the replication of human immunodeficiency virus type 1 (HIV-1), the etiologic agent of acquired immune deficiency syndrome (AIDS), and on the regulatory steps that control the replication of other complex retroviruses that infect human or animal hosts.

Regulatory Proteins of HIV-1

A major interest of the laboratory relates to the mechanism of action of Tat and Rev, the two essential regulatory proteins encoded by HIV-1. Both are critical to HIV-1 replication in culture. The Tat protein is a potent trans-activator of transcription directed by HIV-1's long terminal repeat (LTR) promoter element. The Rev protein, in contrast, acts post-transcriptionally to induce the cytoplasmic expression of a subset of HIV-1 mRNA species that encode the viral structural proteins. Both Tat and Rev are similar, however, in that they act through structured viral RNA target sites, termed TAR in the case of Tat and RRE in the case of Rev.

During the past year, Dr. Cullen's laboratory has used both biochemical and genetic approaches to define sequences within the HIV-1 RRE that are critical for Rev binding. Modification interference analysis was used to delineate a small, ~13-nucleotide RNA sequence within the larger, 234-nucleotide RRE that serves as the primary binding site for Rev *in vitro*. The physiological relevance of these *in vitro* observations was confirmed by the *in vivo* demonstration that a fusion protein consisting of Rev attached to the Tat carboxyl terminus could activate gene expression from an HIV-1 LTR promoter in which critical TAR sequences had been replaced by this minimal Rev-binding site.

The demonstration that Rev could serve to direct the activation domain of Tat to a promoter-proximal RRE-derived RNA target has allowed the genetic dissection of sequences within Tat and Rev that are required for either RNA binding or its subsequent activation. These studies have also demonstrated the existence of a cellular Tat-binding protein that is required for not only Tat function but also TAR binding *in vivo*.

The HIV-1 Nef Protein

The action of the Rev protein divides the temporal pattern of HIV-1 gene expression into an early phase, marked by the expression of the "regulatory" proteins Tat, Rev, and Nef, and a late phase characterized by the high-level expression of the viral structural proteins. While Tat and Rev are both well characterized, Nef has remained an enigma. It is known, however, as a myristoylated, membrane-associated cytoplasmic protein that, in contrast to Tat and Rev, is dispensable for HIV-1 replication in culture. Many isolates of HIV-1, in fact, seem to express Nef proteins that are defective, a problem that has made their functional analysis particularly difficult.

Recently the laboratory of Dr. Ron Desrosiers (Harvard University) showed that Nef was absolutely critical for viral replication and pathogenesis in rhesus monkeys infected with a cloned isolate of simian immunodeficiency virus (SIV). This observation both rekindled interest in the biological role of Nef and provided a Nef isolate that was clearly biologically active.

Analysis of the SIV *nef* gene has demonstrated that Nef profoundly down-regulates the cell surface expression of CD4, the glycoprotein receptor for both HIV-1 and SIV. Nef has no effect on either the rate of synthesis or the level of expression of CD4 in these cells and must, therefore, be blocking cell surface CD4 expression via a post-translational mechanism.

Interestingly, expression of Nef reduces CD4 expression on T cells to such a degree that they are no longer permissive to infection by HIV-1 virions, although a transfected HIV-1 provirus is expressed normally. It is hypothesized that Nef normally functions to facilitate the efficient release of infectious HIV-1 or SIV virions by preventing the premature interaction of viral envelope proteins with CD4 expressed in either the endoplasmic reticulum or on the surface of the infected cell.

HIV-1 Cell Tropism

Isolates of HIV-1 can be divided into two distinct groups based on their ability to infect primary human macrophages. Previously it was shown that this tissue tropism was primarily regulated by the sequence of a small, disulfide-bonded protein loop found within the HIV-1 envelope protein. More recently it has been demonstrated that this so-called V3 loop also regulates the efficiency with which HIV-1 virions in culture are neutralized by soluble, recombinant CD4 protein. Inability to grow in macrophages was tightly correlated with high sensitivity to soluble CD4.

It is believed that CD4 neutralizes HIV-1 virions by inducing a conformational shift in the HIV-1 envelope protein that leads to shedding of the gp120 component. It is therefore hypothesized that the sequence of the V3 loop modulates the nature of this conformational shift, thereby influencing not only the sensitivity of HIV-1 virions to soluble CD4 but also the nature of the interaction of the HIV-1 envelope with specific cell surface proteins subsequent to initial binding of the virion to cell surface CD4.

Gene Therapy Approaches to AIDS

Previously Dr. Cullen's laboratory described mutant forms of the HIV-1 Rev protein that retain the ability to bind to, and multimerize on, the RRE but are unable to function once bound. These mutant Rev proteins are therefore capable of blocking the interaction of functional Rev protein with the RRE and, hence, the replication of HIV-1.

In collaboration with the laboratory of Dr. Gary Nabel (HHMI, University of Michigan), Dr. Cullen's laboratory has now demonstrated that stable expression of such a trans-dominant negative Rev protein in human T cells can render these cells refractory to productive infection by HIV-1. Importantly, these protected T cells displayed a normal growth pattern and retained the ability to elaborate cytokines in response to stimulation. These observations suggest that trans-dominant Rev mutants can protect T cells against HIV-1 without exerting any deleterious effect on the expressing cell. This strategy may therefore represent a promising gene therapy approach to the treatment of HIV-1–infected persons. The gene therapy project described above was supported by a grant from the National Institute of Allergy and Infectious Diseases.

Dr. Cullen is also Associate Professor in the Section of Genetics and the Department of Microbiology and Associate Medical Research Professor in the Department of Medicine at Duke University Medical Center.

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MOLECULAR MECHANISMS IN LYMPHOCYTE DEVELOPMENT

Stephen V. Desiderio, M.D., Ph.D., *Associate Investigator*

Assembly of Antigen Receptor Genes

The antigen receptors of B and T cells exhibit an extraordinary range of binding specificities. This diversity results in large part from a peculiarity of their gene structure: antigen receptor polypeptide chains are encoded in the germline as discrete DNA segments and joined during lymphoid development by site-specific recombination. Antigen receptor gene rearrangement and its regulation remain an area of interest for Dr. Desiderio and his colleagues.

Phosphorylation regulates activity and expression of the recombination activator protein RAG-2. The ability of cells to support rearrangement of antigen receptor genes is absolutely correlated with the expression of the recombination activator genes *RAG-1* and *RAG-2,* but the mechanism by which these genes activate rearrangement is not known. In the past year Dr. Desiderio and his colleagues have found that activity and expression of the RAG-2 protein are regulated by multiple phosphorylation pathways. These observations raise interesting questions concerning the regulation of antigen receptor gene rearrangement by extracellular signals and the coupling of rearrangement to the cell cycle.

To examine the products of *RAG-1* and *-2,* a series of specific polyclonal antibodies were raised and used to immunoprecipitate the proteins from cell lines expressing their genes. The apparent size of the RAG-1 protein was ~110 kDa, as predicted by nucleotide sequence. The RAG-2 protein had an apparent size of 70 kDa, significantly larger than predicted, which suggested that it had undergone posttranslational modification. Both RAG-1 and -2 were found to be phosphorylated and to reside largely in the nucleus.

The RAG-2 protein is 527 amino acids long. By site-directed mutagenesis and phosphopeptide analysis, the major *in vivo* phosphorylation sites were mapped to Ser356 and Ser392. These sites were found to be phosphorylated *in vitro* by purified casein kinase II (CKII).

To examine the functional importance of these CKII-like phosphorylations, mutant RAG-2 proteins carrying Ala substitutions at a series of Ser or Thr sites were assayed for activation of rearrangement *in vivo*. None of these mutations had any effect on expression of *RAG-2* RNA or protein. Substitution of Ala for Ser at position 356 resulted in a large (5- to 10-fold) decrease in activity; the other mutations had no effect on rearrangement. These experiments strongly suggest that the activity of RAG-2 is enhanced by phosphorylation at Ser356 and that CKII, or a CKII-like enzyme, regulates RAG-2 function *in vivo*.

The *RAG-2* sequence shows several potential $p34^{cdc2}$ phosphorylation sites, and two of these were found to be phosphorylated selectively by $p34^{cdc2}$ or a related kinase *in vitro*. Unexpectedly, a mutant carrying an Ala substitution at the major phosphorylation site (RAG-2 T490A) was expressed at a far higher steady-state level than wild type. This effect was not observed upon mutation of the minor $p34^{cdc2}$ phosphorylation site, nor upon mutation of the CKII sites (see above). The relative increase in expression of RAG-2 T490A is most likely the result of protein stabilization, because 1) mutant and wild-type RNA levels were identical and 2) chimeric proteins containing wild-type or mutant RAG-2 polypeptide segments exhibited a similar phenotype. The activities of $p34^{cdc2}$ and related kinases vary during the cell cycle; likewise, RAG-2 protein levels appear to vary, reaching a minimum during S phase. These observations have prompted a specific hypothesis: that phosphorylation of RAG-2 by $p34^{cdc2}$ or a related kinase increases its rate of

degradation. Dr. Desiderio and his colleagues now wish to test this idea and to determine whether their observation represents a specific example of a more general phenomenon.

Recognition of the recombination signal sequences of antigen receptor genes. Assembly of antigen receptor genes is mediated by conserved heptamer and nonamer DNA sequence elements that lie at the sites of recombination. The laboratory has purified a specific DNA-binding protein, NBP, whose binding site coincides with the nonamer recombination signal, suggesting that it is a part of the recombinational machinery. By differential screening of a cDNA expression library with wild-type and mutant nonamer oligonucleotide probes, the laboratory isolated a cDNA clone whose polypeptide product binds DNA fragments containing the nonamer recombinational signal. The function of this polypeptide and its relationship to NBP are now under study. (The project described in this section is supported by a grant from the National Institutes of Health.)

Genetic Basis of Molecular Mimicry by Antibody Molecules

A long-standing hypothesis is that the structures of some antigens may be mimicked by a subset of anti-idiotypic antibodies. Dr. Desiderio and his collaborators Dr. L. Mario Amzel (Department of Biophysics, Johns Hopkins University School of Medicine) and Dr. Pierre M. Ronco (INSERM, Hopital Tenon, Paris) have tested this idea by examining an anti-idiotypic antibody series. An angiotensin II–reactive antibody (Ab1) and an anti-anti-idiotypic antibody (Ab3) are related by an anti-idiotypic antibody (Ab2-β) and were found to have identical antigen-binding properties. The sequences of the variable regions of Ab3 and of Ab1 were nearly identical, even though the Ab1 was raised against a peptide and the Ab3 against a globular protein. Strikingly, amino acid residues that make critical contacts with antigen in the crystal structure of the Ab3-antigen complex are highly conserved in Ab1, indicating that the epitopes of the Ab2-β recognized by Ab3 do indeed resemble the bound structure of the antigen.

Signal Transduction in Lymphocyte Activation

Activation of lymphocytes by antigen and lymphokines is likely mediated by protein-tyrosine phosphorylation, but the tyrosine kinases involved are largely undefined and their functions poorly understood. The answers to such questions remain a major goal of Dr. Desiderio's laboratory.

The B lymphoid–specific protein-tyrosine kinase, Blk. Dr. Desiderio and his colleagues have

identified a new member of the *src* family, *blk*, which is expressed specifically in B lymphoid cells and encodes a 55-kDa protein-tyrosine kinase, Blk. During B cell ontogeny, *blk* expression is closely correlated with expression of two genes, *mb-1* and *B29*, which encode the accessory chains of the B cell antigen-receptor (sIg) complex, suggesting that Blk functions in sIg-mediated signaling. Preliminary experiments suggest a specific physical association between Blk and the product of *B29*, and the structural basis for this association is now under study.

In a separate approach to Blk function, the Desiderio group is constructing lines of transgenic mice that express constitutively active or inactive Blk kinases in B lymphoid cells. If Blk transduces sIg-mediated signals, the resulting mice may exhibit specific defects in B cell development or responsiveness to antigen.

In addition to Blk, several other Src family kinases, including Lyn and Fyn, are expressed in B cells. All Src kinases, and some other molecules, contain a domain (Src-homology region 2, or SH2) that binds phosphotyrosine-containing polypeptides. The group has employed affinity chromatographic methods to identify phosphoproteins whose phosphorylation state changes upon B cell activation and that bind to specific SH2 subsets.

Upon terminal differentiation of B cells to plasma cells, transcription of *blk* ceases. A DNA fragment containing 451 bp of *blk*'s 5' flank was found sufficient to support developmental stage–specific expression of a heterologous gene in B lymphoid cells. By electrophoretic mobility shift assays, two sites within this region were found to bind protein(s) whose activity is correlated positively with *blk* expression. Studies to determine the functional significance of these interactions and to identify the proteins that bind these sites are under way. (The studies described in this paragraph are supported by a grant from the National Institutes of Health.)

The T lymphoid–specific protein-tyrosine kinase Itk. Dr. Desiderio and his colleagues have isolated another novel lymphoid-specific tyrosine kinase gene, *itk* (IL-2 [interleukin-2]-inducible T cell kinase). Conceptual translation of the *itk* sequence predicts a 72-kDa tyrosine kinase of unusual structure: while it is related to members of the Src family, it lacks the amino-terminal myristoylation consensus sequence and the regulatory, carboxyl-terminal tyrosine residue characteristic of Src kinases. Antibodies elicited by Itk-specific peptides detect a 72-kDa phosphoprotein in thymus and cultured T cells. As predicted, this protein is associated with protein-tyrosine kinase activity.

Expression of *itk* is restricted to the T cell lineage,

suggesting that the tyrosine kinase encoded by *itk* functions in a signal transduction pathway unique to T lymphocytes. RNAs for *itk* and for the α chain of the IL-2 receptor are co-induced by the T cell mitogen IL-2. By 2 hours after IL-2 administration, the steady-state level of itk RNA increased eightfold, implicating *itk* in the response of T cells to this lymphokine.

Dr. Desiderio is also Associate Professor of Molecular Biology and Genetics at the Johns Hopkins University School of Medicine.

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TRANSCRIPTION CONTROL DURING EARLY *DROSOPHILA* DEVELOPMENT

Claude Desplan, Ph.D., *Associate Investigator*

The goal of Dr. Desplan's laboratory is to understand the molecular mechanisms involved in the regulatory interactions among developmental genes. In light of the complexity of the system, a productive approach is, first, to characterize the molecular interactions *in vitro* and, second, to design *in vivo* systems to test models of regulatory interactions consistent with properties uncovered through the first step. Dr. Desplan and his colleagues are investigating the events leading to patterning in two major systems in the *Drosophila* embryo. One involves the establishment of the early anterior pattern, while the other is a structure-function analysis of genes that control later steps of development.

Dimerization and DNA-binding Specificity of the Homeodomain

Most developmental processes involve genes that encode a homeodomain (HD). The HD includes a domain similar to the helix-turn-helix motif present in many prokaryotic DNA-binding transcriptional regulators. The analyses by Dr. Desplan and his colleagues of the HD *in vitro* and in cell culture have led them to propose that the specificity changes among classes of HDs are due to the nature of a single amino acid at position 9 of the recognition helix. A powerful selection procedure from a library of random sequences has led to the discovery that at least some HDs are able to dimerize cooperatively on palindromic sites of DNA.

The geometry of this dimerization, which is sur-

prising in view of the published structures of HDs, is regulated by residue 9 of the recognition helix. Changing this position can change not only the base pairs recognized by the HD but also the spacing requirement between the two halves of the palindrome. Also, when residue 9 is a lysine, as in Bicoid, the HD binds with high specificity as a monomer and does not appear capable of cooperative dimerization. These observations are important, because they suggest that various HDs form heterodimers, a process that may help explain some of the combinatorial interactions observed between segmentation genes.

The *paired* Gene Encodes a Multifunctional Transcription Factor

In addition to an HD, the product of the *paired* gene, Prd, contains domains that are conserved in other developmental gene products. The Paired domain also mediates DNA binding, making Prd a bifunctional transcription factor. Although both the Paired domain and the HD can bind to DNA independently, they can also bind cooperatively to adjacent sites when both are present in the same molecule.

The cooperation between the two motifs may refine the functional specificity of genes containing highly related domains. An *in vivo* structure-function analysis of the product of *prd* is an attempt to correlate the multiple DNA-binding functions of Prd defined *in vitro* with the multiple genetic functions of the *prd* gene, as a regulator of

segmentation genes and of genes in the nervous system. This is achieved by testing the ability of versions of the *prd* gene, modified in regions encoding each of the subdomains of the Prd protein, to rescue some of the differential molecular phenotypes of *prd⁻* embryos.

From Segmentation to Organogenesis

BK27 is a gene highly homologous to *prd* but which appears to be involved in the early steps of organogenesis. This gene is first expressed in a group of cells that represent the progenitor of the salivary gland placode, at a stage when no tissue differentiation has occurred. This expression is controlled by positional cues from the dorsoventral and anteroposterior determinants. As the expression of this gene precedes morphological events, it provides a model of transcriptional commitment to a particular differentiation pathway. Presently mutants are being generated in this gene to analyze the expression of putative target genes and the resulting morphological phenotype.

Because the protein encoded by *BK27* has an organization very similar to that of Prd but has a Prd domain from a divergent class, its product will also be very useful in dissecting the residues that determine the specificity of the Prd domain.

Down-regulation of the Morphogen Bicoid by the Torso Receptor-mediated Signal Transduction Cascade

Most aspects of anterior body pattern are specified by a graded distribution of the Bicoid protein (Bcd), which is presumed to bind with different affinities to subordinate regulatory genes, leading to their transcriptional activation in distinct anterior domains. However, transcription of these target genes is subsequently repressed at the anterior pole in response to the local activation of the receptor tyrosine kinase *torso* (*tor*). Both activation by *bcd* and repression by *tor* can be reproduced with an artificial promoter consisting solely of *bcd*-binding sites placed upstream of a naive transcriptional start site.

Repression depends critically on the function of the *Drosophila* homologue of the serine-threonine kinase *raf* (D-raf), but not on the two transcription factors whose local activity was previously thought to constitute the sole transcriptional effect of the *tor* signaling system. Thus the activity of Bcd protein appears to be down-regulated by input from the *tor* signaling cascade, possibly by D-raf–dependent phosphorylation of the structural domain normally required for activating transcription.

Applications to the National Institutes of Health and the American Cancer Society are pending for support of this part of the research program.

Autoregulation of *hunchback*

The expression of *bcd* targets was previously thought to depend on Bcd alone. However, the product of another maternal gene, *hunchback* (*hb*), appears to be required for full synergistic activation with *bcd*. An Hb protein binding site exists in close association with a Bcd site in the zygotic *hb* promoter and may be required for *hb* activation. The contribution of the maternal component of *hb* to anterior patterning has implications for the evolution of *bcd* and *hb* morphogenetic functions. A cluster of Hb binding sites also exists in another region of the *hb* promoter that controls *hb* autoregulation at a later stage in development.

Applications to the National Institutes of Health and the American Cancer Society are pending for support of this part of the research program.

These studies have led Dr. Desplan and his colleagues to investigate both the general transcriptional functions carried out by specific developmental proteins and the particular properties of each gene product. The combinatorial effects of such factors both *in vitro* and *in vivo* is now being investigated to acquire insight into the mechanisms controlling the coordinate expression of developmental genes.

Dr. Desplan is also Associate Professor and Head of the Laboratory of Molecular Genetics at the Rockefeller University.

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IMMUNE EVASION BY PARASITES THAT CAUSE TROPICAL DISEASES

JOHN E. DONELSON, PH.D., *Investigator*

More than a billion people in tropical areas of the world endure parasitic infections throughout most of their lives. The protozoan and helminthic parasites that cause these infectious diseases possess sophisticated molecular mechanisms for evading the immune response. Most of these immune evasion strategies are based on the appearance of unique proteins on the surface of the parasite at specific times during infection. Dr. Donelson's laboratory seeks to understand at the genetic level how parasites regulate the production of these surface proteins during their different developmental stages. The information gained from the studies should contribute to eradication or better control of these diseases.

Antigenic Variation by African Trypanosomes

African trypanosomes are protozoan parasites that cause sleeping sickness throughout equatorial Africa. They are transmitted from tsetse flies to the bloodstream, where they continually confront the humoral and cellular immune systems as they grow and multiply. Each trypanosome in the bloodstream contains on its surface about 10^7 copies of a single protein called the variant surface glycoprotein, or VSG. The trypanosome population survives the continuous immune assault against it because individual parasites occasionally switch spontaneously from the expression of one VSG to another—a process called antigenic variation. After a switch the changed parasite and its descendants remain temporarily "one step ahead" of the host defenses while a new immune response is mounted. The trypanosome genome contains about 1,000 different genes encoding antigenically distinct VSGs.

Usually only one VSG gene at a time undergoes transcription. Rearrangements and duplications of these genes are partly responsible for the selection of which VSG gene is to be transcribed. The rearrangements maneuver specific VSG genes into and out of telomere-linked "expression sites" where transcription occurs. The process is complicated by the fact that several such expression sites exist in the genome, yet only one is normally activated at a time. Dr. Donelson's laboratory is currently examining a subset of 12 VSG genes that are expressed specifically at the metacyclic stage, which is the final developmental stage in the tsetse fly. These VSG genes are always adjacent to telomeres and have several unique characteristics that contribute to their expression during this specific developmental stage.

Nuclear run-on experiments have shown that transcription initiation of these metacyclic VSG genes occurs at developmental stages that do not possess a VSG, suggesting that the high steady-state levels of these transcripts at the metacyclic stage are regulated by post-transcriptional events. Placement of sequences flanking these metacyclic VSG genes into a plasmid containing a luciferase reporter gene, followed by DNA transfections of the recombinant plasmid, have shown which of the flanking DNA segments are necessary for the proper appearance of the VSG. Proteins that bind to these regulatory regions are now under investigation.

Another distinctive feature of telomere-linked VSG genes is their high rate of mutation. Careful examination of four different bloodstream trypanosome clones that reexpress a specific metacyclic VSG via a gene duplication mechanism revealed that each of these trypanosome clones possesses a VSG containing a slightly different amino acid sequence. Recombinant cloning of the original VSG gene and its duplicated, expressed gene copy demonstrated that the duplicated VSG gene undergoes point mutations during the gene conversion (i.e., duplicative transposition) event.

Additional examination of several VSG genes expressed only in the bloodstream demonstrated that, unlike the metacyclic VSG genes, they do not accumulate point changes. Thus the metacyclic VSG genes appear to be in a distinctive chromosomal environment that tolerates and/or facilitates point mutations. Such changes within the VSG genes would contribute to a greater diversity of the 12 VSGs, enhancing the phenomenon of antigenic variation at the metacyclic developmental stage.

Expression of Genes for a Surface Protease of Leishmania

Leishmania parasites are protozoan organisms that are transmitted as promastigotes by sand flies. Depending on the *Leishmania* species, they cause either visceral, cutaneous, or mucocutaneous leishmaniasis. These parasites have a different mechanism of evading the immune response than the African trypanosomes. They are intracellular pathogens that reside inside macrophages—cells of the immune system whose normal function is to engulf and destroy foreign pathogens and substances. In collaboration with Dr. Mary Wilson of the Department of Internal Medicine at the University of Iowa, Dr. Donelson's laboratory is examining how leish-

mania parasites invade macrophages and survive within their hostile oxidative environment.

One important parasite constituent in both the entry and survival process is a protease called glycoprotein 63 (gp63). This is the most abundant protein on the surface of stationary-phase promastigotes, which can invade macrophages, but is nearly absent from log-phase cells, which do not infect macrophages. Although log-phase promastigotes possess very little gp63, they have as much steady-state RNA for gp63 as do stationary promastigotes.

Characterization of gp63 cDNAs from log- and stationary-phase promastigotes revealed three distinct transcripts for gp63, each of which encodes a different form of the protease. One transcript of 2.7 kb occurs in log cells; another of 3.0 kb, in stationary cells; and a third of 3.1 kb, a transcript of low abundance, is constitutively expressed. Cells that are intermediate between log and stationary phase contain all three transcripts. Each of the transcripts, which differ primarily in the length and sequences of their 3'-nontranslated regions, directed the synthesis of gp63 in a heterologous *in vitro* translation system, suggesting that mechanisms other than transcription regulate the gp63 levels.

The gp63 RNAs are products of two clusters of genes. Nuclear run-on experiments showed that transcription initiation from all three gene clusters occurs continuously and that the steady-state levels of the three transcripts are regulated by post-transcriptional events. These post-translational regulatory processes occur, at least in part, when a leader of 39 nucleotides is spliced onto the 5' ends of the gp63 transcripts. In addition, DNA transfections of plasmid constructs containing the different 3'-untranslated regions downstream of a luciferase gene suggest that the unique 3' nontranslated sequences also influence the stabilities of the transcripts at different promastigote growth stages.

Other experiments are in progress to examine the effect of deleting or altering the gp63 genes on the infectivity and survival of the leishmania parasite in macrophages. Although not completed, the work demonstrates that the regulation of gp63 levels in leishmania promastigotes occurs at several steps. Parts of this research are supported by a grant from the National Institutes of Health.

Dr. Donelson is also Distinguished Professor of Biochemistry at the University of Iowa College of Medicine, Iowa City.

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MOLECULAR GENETICS OF HEREDITARY HEARING LOSS

Geoffrey M. Duyk, M.D., Ph.D., *Assistant Investigator*

Hearing loss is the most common form of sensory impairment. Profound childhood hearing loss has an incidence of 4–8 per 1,000 births in developed countries. In at least half of the cases, the likely etiology is a single gene mutation. It is estimated that as many as 5% of school-age children have unilateral and/or mild-to-moderate hearing loss, representing a potentially reversible cause of learning difficulty. In addition to early-onset hearing loss, progressive hearing loss, or presbycusis, occurs as part of the normal aging process. By the time we reach the age of 65, there is a one-in-six chance of having functionally significant hearing loss.

While environmental causes such as acoustic trauma, infection, or ototoxic drugs play a significant causative role in auditory sensory impairment, underlying predisposing genetic factors are likely to be identified. Some types of genetic hearing loss can easily be distinguished, since they occur as part of a recognizable syndrome. Hearing loss is a major component in more than 100 defined genetic disorders.

Nonsyndromic, or undifferentiated, hearing loss represents the second major category of hearing loss in which auditory sensory impairment is an isolated finding. Analysis of affected families is complicated by the difficulty in determining whether the hearing loss in the members is genetic or acquired. If the hearing loss is proved to be hereditary, analysis of pedigrees may be further complicated by genetic heterogeneity and nonassortative mating. The broad goal of Dr. Duyk's laboratory is to develop methods and resources to identify and study the genetic basis of nonsyndromic hearing loss.

Genetics

An important aspect of the laboratory's research is the identification of pedigrees that segregate hearing loss and are also suitable for linkage analysis. The genetic analysis of hearing loss in human populations is complicated by the fact that many of these syndromes are not readily differentiated from one another (genetic heterogeneity) and the fact that individuals with profound hearing loss often intermarry (nonassortative mating).

In collaboration with the Massachusetts Eye and Ear Infirmary (ME&EI), Dr. Duyk and his colleagues have identified two kindreds affected with an autosomal dominant, progressive sensorineural hearing loss. The primary pathologic change appears to be deposition of mucopolysaccharide-containing ground substance in the cochlear and vestibular nerve channels that causes strangulation and subsequent degeneration of the dendritic nerve channels. Julie Gastier (Department of Genetics, Harvard Medical School) and Dr. Umang Khetarpal (ME&EI) have initiated a study of this family, with a view to mapping the location of the gene and eventually identifying its molecular defect.

In the study of hereditary hearing loss, pedigrees suitable for conventional linkage analysis are the exception rather than the rule. To analyze families with nonsyndromic hearing loss that is most likely due to autosomal recessive loci, the alternative approach of homozygosity mapping of affected individuals in consanguineous pedigrees will be utilized, in conjunction with conventional linkage analysis of nuclear families with multiple affected siblings. The use of homozygosity mapping with affected offspring of first cousins is a much more efficient approach than reliance on small nuclear nonconsanguineous pedigrees, since far fewer families may be expected to demonstrate linkage. This strategy involves looking for homozygosity of DNA variants in affected children from consanguineous unions on the premise that the region adjacent to the disease locus will be homozygous by descent. Such approaches will identify candidate regions within the genome likely to encode a gene for nonsyndromic hearing loss. The feasibility and power of such approaches will dramatically improve with the increasing density of the human genetic map and the availability of highly informative polymerase chain reaction (PCR)-based short tandem-repeat polymorphisms (STRPs).

Toward this end, Dr. Duyk's laboratory has developed an efficient method (marker selection) for the construction of small insert genomic libraries highly enriched for selected classes of short tandem repeats (STRs). These libraries will aid in the production of high-resolution genetic maps composed of highly polymorphic, homogeneously distributed PCR-based markers. Members of Dr. Duyk's laboratory (Jacqueline Pulido, Julie Gastier, and Wendi Beck) are developing STRP markers based on the large numbers of trinucleotide and tetranucleotide repeats isolated by improved versions of this method. In collaboration with Drs. Jeffrey Murray and Val Sheffield (University of Iowa), Dr. Kenneth Buetow (Fox Chase Cancer Center), and Dr. James Weber (Marshfield Clinic), the laboratory will as-

semble these markers into a high-resolution human genetic linkage map, with a predicted minimum sex-averaged resolution of 2.5 cM.

Candidate Genes and Mechanosensory Transduction

It is unlikely that localization of genes for hearing loss by homozygosity mapping and related approaches will suffice for positional cloning of the targeted genes, but the integration of candidate genes into the genetic map should facilitate their recovery. As the signal transduction pathways required for the development of the inner ear begin to be understood and the components of the auditory sensory transduction system are defined at a molecular level, the list of potential candidate genes will grow.

In collaboration with Dr. David Corey (HHMI, Massachusetts General Hospital), Dr. Duyk and his colleagues have begun the process of identifying the molecular components of the hearing apparatus by combining molecular biological and biophysical approaches. Their starting point has been the construction of cDNA libraries from microdissected inner ear material highly enriched for hair cells. Using these resources, Dr. Charles Solc and Vida Meyers have identified members of the myosin gene family that are expressed in hair cells as candidates for an essential component of the auditory mechanosensory process, the adaptation motor. Dr. Hong Chen has used interaction cloning strategies to identify additional candidate components of the sensory apparatus. In collaboration with Ici Thalmann (Washington University, St. Louis), he has also cloned an organ of Corti–specific gene, based on partial amino acid sequence. The continued study of these compo-

nents and others will lead to the understanding of the molecular basis of hearing.

As components of the sensory transduction pathway are identified, human and murine homologues will be recovered and mapped to specific chromosomes, and closely linked DNA polymorphic markers will be developed and integrated into the high-resolution genetic map. This information will represent important resources in the hunt for genes underlying hereditary hearing loss.

Dr. Duyk is also Assistant Professor of Genetics at Harvard Medical School and a member of the Eaton-Peabody Laboratory at the Massachusetts Eye and Ear Infirmary.

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TUMOR SUPPRESSOR GENES

ANDREW P. FEINBERG, M.D., M.P.H., *Associate Investigator*

Twenty years ago, Knudson proposed that some cancers arise by two genetic "hits," the first transmitted as a dominant trait for hereditary predisposition to cancer and the second arising in somatic cells. These two hits, if allelic, would represent a recessive, or suppressor, tumor gene. Two of Knudson's paradigms were retinoblastoma and Wilms' tumor (WT), since they sometimes arise as multiple tumors in very young children. Presumably a germ-line mutation affecting all of their cells represents the first "hit." While the retinoblastoma gene ele-

gantly fits Knudson's model, Dr. Feinberg's laboratory has found that WT is far more complex, involves multiple genes, and may also involve the unconventional pathogenetic mechanism of genetic imprinting.

Wilms' Tumor Genes on 11p13

Some children with the WAGR (Wilms' tumor, aniridia, genitourinary dysplasia, and mental retardation) syndrome show overlapping deletions involving 11p13, and sporadically occurring WTs were

also shown cytogenetically to lose this band. Several years ago Dr. Feinberg's laboratory first applied RFLPs (restriction fragment length polymorphisms) to show loss of allelic heterozygosity of 11p in WT, molecular evidence for a tumor suppressor gene on this chromosome. In collaboration with Drs. David Schlessinger (Washington University, St. Louis) and Bryan Williams (Hospital for Sick Children, Toronto), the laboratory cloned the 11p13 WT gene region in yeast artificial chromosomes (YACs).

Interestingly, they found that this region contains at least two genes (*WIT-1* and *WIT-2*) expressed specifically in developing kidney. *WIT-2* (also known as *WT1*) is a transcription factor that is mutated in some WTs. These mutations occur infrequently, however, and the laboratory is investigating whether reduced expression of the gene may be a more common mechanism for tumorigenesis. Both *WIT-1* and *-2* showed reduced or absent expression in ~30% of sporadically occurring WTs. These tumors are of a particular histologic type that reflects the earliest stages of renal development, and WTs in WAGR patients are exclusively of this type.

An Additional Wilms' Tumor Gene on 11p15

One of the predictions of Knudson's model is that some families should develop Wilms' tumor as an autosomal dominant trait, by transmitting one mutant copy of the gene. Previously Dr. Feinberg's laboratory mapped such a disorder, Beckwith-Wiedemann syndrome (BWS), to 11p15, and not 11p13 (site of *WT1*), by genetic linkage analysis. BWS is characterized by multiorgan overgrowth, other developmental malformations, and predisposition to a variety of "embryonal" tumors, such as WT, rhabdomyosarcoma, and liver and adrenal tumors. Supporting the idea of genetic heterogeneity in WT, BWS-associated tumors, in contrast to WAGR-associated tumors, are of a histologic type reflecting later stages of renal development. They also show normal *WT1* expression.

In collaboration with Drs. Marcel Mannens and Jan Hoovers (University of Amsterdam), the laboratory has found that several germline balanced chromosomal rearrangements in BWS patients lie within a small region of 11p15. To localize this gene, the laboratory has isolated 23 YACs from 11p15. These were found to include six of the chromosomal breakpoints from BWS patients. The breakpoints lie within a 1-Mb region, and the laboratory is now looking for candidate genes within the YACs spanning them.

One of the most intriguing aspects of BWS is the possible role of genetic imprinting (allele-specific modification). At least two mouse homologues of 11p15 genes are imprinted, and some BWS patients show paternal uniparental disomy for most of 11p15, suggesting a difference between maternal and paternal alleles. The laboratory found that six of six balanced germline rearrangements were maternally derived, and six of six unbalanced duplications of 11p15 were paternal in origin, also consistent with an imprinted gene.

A Novel Strategy for Isolating Tumor Suppressor Genes

Although the *WT1* gene is on 11p13, Dr. Feinberg and his colleagues found that genetic loss in WT, as reflected by loss of allelic heterozygosity, specifically involves 11p15, site of the BWS gene, rather than 11p13. Furthermore, 11p15 is also commonly lost in other embryonal tumors and in ovarian, lung, and breast cancers. Thus WT is more complex than investigators had previously believed, and an 11p15 gene may also be important in the progression of common cancers.

A fundamental problem in the identification and isolation of tumor suppressor and other growth-inhibiting genes is the fact that one loses the power of genetic complementation at the subchromosomal level. Thus, while the existence of tumor suppressor genes was first demonstrated by cell fusion—and suppression can also be detected by monochromosome transfer into tumor cells—direct expression cloning of suppressor genes in manageable vectors is usually not possible, since suppression is normally selected against. Furthermore, while YACs have been transferred to mammalian cells, success has been limited to small genes and specific cell types, and assaying for tumor suppression with the thousands of YACs needed for a chromosome is impractical. The laboratory therefore sought to develop a strategy for transferring subchromosomal fragments intermediate in size between YACs and chromosomes.

This strategy involves three steps: 1) transfection of a mammalian selectable marker gene into mouse cells containing a single independently selectable human chromosome; 2) chromosome transfer by microcell fusion followed by double selection for both the human chromosome and the marker gene, yielding a panel of hybrids that carry the human chromosome with the marker gene integrated randomly within it; and 3) irradiated microcell transfer of the pooled hybrid panel to isolate individual marker-containing chromosomal subfragments from the remainder of the chromosome. The resulting "subchromosomal transferrable fragments," or STFs, unlike conventional radiation hybrids, can then be transferred individually to any mammalian

cell of choice by virtue of the selectable marker gene within them. STFs might also eventually prove useful for cloning other types of genes, such as those involved in cellular aging, since they do not require a selective growth advantage in complementation experiments. (This work was supported in part by the National Institutes of Health.)

The laboratory constructed a library of STFs from 11p15. Several of these caused *in vitro* growth arrest of WT and rhabdomyosarcoma cells, demonstrating that tumor suppressor genes can be isolated and transferred in this manner. The tumor suppressor activity was localized within a region of 2 Mb adjacent to, but apparently distinct from, the BWS chromosomal breakpoints. The laboratory is now looking for genes in this region and determining whether it might represent yet a third 11p WT-related locus.

Finally, WT is made even more complex by the fact that some non-BWS families with WT do not show linkage of this trait to chromosome 11. Recently, Dr. Feinberg's group, with a former sabbatical member of the laboratory, Dr. Anthony E. Reeve (University of Otago, New Zealand), found involvement of chromosome 16 in WT.

Dr. Feinberg is also Associate Professor of Internal Medicine and Human Genetics at the University of Michigan Medical School.

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HUMAN MOLECULAR GENETICS AND COMPARATIVE GENE MAPPING

Uta Francke, M.D., *Investigator*

The mapping of genes on human and mouse chromosomes has been a long-standing activity in Dr. Francke's laboratory. With focus on cloned genes of known function that are expressed specifically in the nervous system or other differentiated tissues, mapping results are obtained that are relevant in different respects. Comparative mapping of chromosomes within and between species reveals insight into genome evolution and leads to the detection of new members of gene families. Mapping information is used to define candidate genes for human inherited disorders or phenotypic mutations in mice. Furthermore, understanding of the phenotypic consequences of chromosome aneuploidy is facilitated by knowledge of specific genes located in the aneuploid segment.

By Southern hybridization or polymerase chain re-action (PCR) analysis of somatic cell hybrid panels with defined subsets of human and mouse chromosomes, and by *in situ* hybridization of cloned genes to chromosomes using fluorescence detection methods, as well as by genetic mapping with recombinant inbred strains of mice, the laboratory has determined the chromosomal map positions for numerous genes in both species.

Mapping Studies Identify Disease Genes

The gene for a peripheral myelin protein (PMP-22), which had been independently isolated as a growth-arrest gene, was assigned to mouse chromosome 11 and human chromosome 17p. By virtue of these localizations and the gene's function in the peripheral nervous system, it became a candidate for involvement in the mouse mutation *Trembler*

and in the human hereditary motor and sensory neuropathy Charcot-Marie-Tooth disease, type 1A (CMT1A). Point mutations could indeed be demonstrated in two strains of *Trembler* mice.

In human CMT1A patients, the PMP-22 gene is located on a submicroscopically duplicated segment of 17p without any evidence for its disruption. Thus the gene may be involved in both the human and the mouse mutations, but the pathophysiological mechanisms must be different. While amino acid substitutions in an important part of the protein are likely to be responsible in mice for the functional disruption, in humans the presence of an extra copy of an apparently undisrupted gene appears to result in a similar peripheral neuropathy phenotype.

The gene for the small nuclear ribonucleoprotein polypeptide N (SNRPN) that is predominantly expressed in brain and plays a role in alternative splicing of calcitonin was mapped to two different chromosomes, in both humans and mice. The intron-containing, presumably functional genes were mapped to known homologous regions on human chromosome 15 and mouse chromosome 7, while the genes on the other two chromosomes represent processed pseudogenes.

The Prader-Willi syndrome (PWS), characterized by congenital hypotonia, hypogonadism, mental retardation, and obesity due to lack of appetite control, is often associated with a small deletion of region 15q11.2-q13. By studying patients with this syndrome, the SNRPN gene was mapped to the deletion interval critical for PWS. This is the first gene of known function to be mapped into the smallest deletion overlap region.

It is well established that genes contributing to this phenotype are maternally imprinted. In PWS deletion patients, the deleted chromosome is paternally derived. In PWS patients without the deletion, two maternally derived chromosomes are present (uniparental disomy).

Since the SNRPN gene is predominantly expressed in the brain, and human fresh brain from these patients is not easily available, studies were conducted in the mouse. They indicate that the SNRPN gene is indeed imprinted on the maternally derived chromosome. These findings support the hypothesis that this gene contributes to the deletion phenotype. Since no rearrangements or deletions of the gene were found in cases of atypical PWS, it is suggested that PWS is truly a microdeletion syndrome in which multiple genes are involved. The SNRPN gene is currently used as an anchor to isolate YACs from this region in which additional expressed genes will be identified and studied for uniparental expression.

Mechanisms of Chromosome Translocations

Two studies completed in the past year addressed the question of the origin of chromosome translocations at the molecular level. A paternally derived t(X;4) translocation with a breakpoint disrupting the dystrophin gene was identified in a female with Duchenne muscular dystrophy. The translocation chromosomes were isolated in somatic cell hybrids, and the region involved in the translocation was cloned from both translocation chromosomes and normal homologues.

When the sequences were compared, it became apparent that the translocation was associated with deletion of ~ 5 kb from the X chromosome. Furthermore, a conserved sequence motif was detected exactly at the three breakpoints. This observation suggests a possible mechanism for this complex rearrangement that involves juxtaposition of the common sequence motif at the three sites and sequence-specific breakage and recombination between nonhomologous chromosomes. The results also illustrate that translocations that appear balanced at the microscopic level may be associated with the deletion of a significant amount of DNA when studied at the molecular level.

In transgenic mice, microinjection of a cDNA construct containing the rat cDNA for peripheral myelin protein P_0 and integration of 50 copies of construct into the mouse genome resulted in reduced litter sizes. A reciprocal translocation between chromosomes 1 and 14 was identified and characterized in embryos. Clusters of transgenes were localized by fluorescence *in situ* hybridization on both translocation chromosomes in the vicinity of the breakpoints. The endogenous P_0 gene was mapped to chromosome 1 near the translocation breakpoint but was shown not to be disrupted by the translocation event. These observations suggest models by which the insertion of the transgene array may have directly contributed to the translocation event.

Mutations Causing Growth Hormone Insensitivity Syndrome (GHIS)

This rare autosomal recessive disorder is characterized by severe growth retardation of postnatal onset, normal or elevated serum growth hormone levels, decreased serum levels of insulin-like growth factor I, lack of clinical response to exogenous growth hormone, and absence of high-affinity serum growth hormone–binding activity. Dr. Francke's laboratory has continued to look for mutations in the growth hormone receptor (GHR) gene in this disorder. All but 1 of 56 affected individuals from southern Ecuador were found to be homozygous for

a single base substitution in exon 6 that generates a new 5' splice site that is used exclusively and results in an mRNA deleted for 24 bases.

The search for this novel mutation in patients from other areas of the world had been negative. During the past year seven unrelated patients from four different continents, including South African blacks and American blacks, were studied, and six different mutations that account for all 14 alleles were identified. These include two new splice site mutations, two different dinucleotide deletions, and one new and one previously reported nonsense mutation. The mutations appear to be unique to particular geographic areas, and most patients are homozygous for the same alleles.

All of the 10 mutations identified worldwide (6 of them discovered in Dr. Francke's laboratory) involve the extracellular domain and are predicted to lead to virtual absence of the receptor from the cell surface. Missense mutations in the extracellular domain that may lead to abnormal receptor interactions, or mutations in the intracellular domain that may interfere with signal transduction, have not yet been identified. It is possible that such mutations would result in a milder form of dwarfism.

Dr. Francke is also Professor of Genetics and Pediatrics at the Stanford University School of Medicine and Medical Staff member of Lucile Salter Packard Children's Hospital and Stanford University Hospital.

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GENETICS OF OBESITY AND TYPE II DIABETES

JEFFREY M. FRIEDMAN, M.D., PH.D., *Assistant Investigator*

The assimilation, storage, and disposition of nutrient energy constitute a complex homeostatic system central to the survival of metazoa. In vertebrates and particularly among land-dwelling mammalian species, the ability to store large quantities of food in the form of adipose tissue triglycerides is crucial for surviving long periods of food deprivation. To maintain such food stores without sustaining continual alterations in the size and shape of the organism, a balance between energy intake and expenditure must be achieved. Despite intensive investigation, the molecular mechanisms that regulate energy intake and energy expenditure remain to be elucidated. It is anticipated that the identification of molecules that transduce nutritional information and regulate these functions will be critical to the understanding of the regulation of body weight in health and in disease states such as cancer.

Detailed metabolic and behavioral studies in humans and other mammals have suggested that specific central and peripheral neural circuits sense and react to both the overall nutritional state and recent food intake of an organism. From this work a hypothesis has emerged that posits a "set point" for determining how much the individual should weigh. Deviations in weight from the set point result in compensatory changes in food intake and energy expenditure that generally return the individual's weight to some genetically determined level.

This set point hypothesis predicts that the levels of peripherally synthesized molecules (hormones perhaps) reflect the nutritional state of an individual and that these levels, these "satiety factors," are sensed by feeding control centers in the hypothalamus and elsewhere. The identity of satiety factors, however, remains to be elucidated. Dr. Friedman's laboratory is taking a genetic approach in the analysis of factors involved in the control of feeding behavior. This involves ongoing attempts to clone several rodent obesity genes: *obese (ob)*, *diabetes (db)*, *fat*, and *tubby (tub)*. To date, the most intensive efforts have been aimed at the molecular cloning of the recessive *ob* and *db* mutations that result in profound obesity. (This work is funded by the National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health.)

The obesity in these mutant animals is the result of abnormalities in feeding behavior and energy ex-

penditure and may reflect a genetic defect in the set point. Since the animals become profoundly diabetic, their investigation also allows the dissection of the genes that play a role in the development of type II diabetes, which is often associated with obesity. More recently, efforts have begun to map and ultimately clone the *fat* and *tubby* mutations.

Molecular Cloning of the Mouse *ob* and *db* Genes

In 1950, during the course of experiments aimed at generating inbred mouse lines, Dr. George Snell (Jackson Laboratories) identified a recessive mutation, *obese* (*ob*), that resulted in massive obesity. Mutant animals often weigh three times as much as their lean litter mates at adulthood. Subsequent genetic studies localized this mutation to mouse chromosome 6.

In 1964 Dr. Douglas Coleman identified a second mutation, *diabetes* (*db*). When bred on the same genetic background, this mutation, which was genetically localized to mouse chromosome 4, has a phenotype identical to *ob*. On the basis of studies employing parabiosis (crossed circulation studies) between obese and non-obese animals, Dr. Coleman concluded that animals carrying the *ob* mutations lacked a circulating appetite-suppressing factor and that animals with the *db* mutation lacked a receptor for this satiety factor. It has proved difficult, however, to identify the *ob* and *db* gene products directly, since the profound obesity of the animals results in numerous secondary endocrine and biochemical abnormalities.

To isolate the *ob* and *db* genes, large crosses segregating the mutations between *Mus castaneus* mice and the two mutant strains, C57BL/6J *ob/ob* and *db/db*, have been completed and the progeny have been characterized. More than 1,000 meiotic events have been scored. These crosses have proved valuable for the genetic mapping of both *ob* and *db* and have suggested an approach to the identification of genes that are responsible for the animals' type II diabetes.

In these genetic crosses the phenotype of the mutation is influenced by genes from the *Mus castaneus* counterstrain to which the *ob* and *db* mice are bred. In general these variant alleles from the counterstrain tend to make *ob/ob* and *db/db* animals more diabetic. Careful measurement of plasma [glucose] and [insulin] among these progeny has not only ensured that correct assignment of genotype at the *ob* and *db* loci has been made but has also permitted identification of animals with severe diabetes, which is never seen among the progenitor C57BL/6J *ob/ob* or *db/db* animals. Thus the levels of plasma

[glucose] and [insulin] have become important in their own right, since their distribution among the progeny of these crosses has suggested that ~80% of the variance in plasma [glucose] and [insulin] is genetic.

The mathematical and analytical tools are currently available to dissect this genetic variance and localize the relevant genes. This strategy, which is currently being implemented, seeks to identify restriction fragment length polymorphisms (RFLPs) that are of the same haplotype as the counterstrain among the diabetic animals but are of the C57BL/6J haplotype in the nondiabetic animals. In such instances it can be deduced that the RFLP is linked to a gene that predisposes an animal to type II diabetes.

The generation of crosses of the size referred to above also allows the compilation of very fine genetic maps that will facilitate the cloning of these mutant genes. Detailed genetic maps of the region around the mutations have previously been generated. The gene order in the vicinity of *ob* is *met–irp*–CF (cystic fibrosis)–*ob*–CPA (carboxypeptidase A)–TcRβ (T cell receptor-β). CF and CPA flank *ob* and are ~4 cM apart. RFLPs detected by each of these genes are designated *met*, *irp*, CF, and CPA. The gene order around *db* is *ifα–c-jun–db–urod–D4Rp1–glut1*.

More recently several new RFLPs have been identified from libraries made by chromosome microdissection. The use of these libraries and a chromosome-specific library for chromosomes 4 and 6 has also led to the compilation of a dense genetic map of these two chromosomes. (This work is funded by a grant from the National Institutes of Health.)

These libraries have also yielded probes in close proximity to the *ob* and *db* mutations. In the case of *ob*, a new RFLP, D6Rck13, has been isolated that has not recombined with *ob* mice in 900 meioses. This suggests that *ob* has been localized to a region of ~500 kb. In the case of *db*, two distal probes are available that are both <0.4 cM from the mutant gene. A proximal marker has also been identified that is 0.5 cM from *db*. These probes can be used as starting points with which to initiate chromosome walking experiments using yeast artificial chromosomes (YACs). YAC clones have been isolated for all of the new probes as well as for CPA (1 cM from *ob*). Five independent YACs have been identified for the D6Rck13 microclone that is nonrecombinant with *ob*.

In separate experiments with Dr. Rudolph Leibel, another mutation, the *fatty* rat mutation (*fa*), was segregated in crosses with +/+ Brown Norway rats. Genetic linkage analysis of the progeny revealed that the *fatty* mutation is flanked by the same RFLPs

as *db*: *ifα* and *glut1*. These data strongly suggest that the *db* mouse and the *fa/fa* rat have defects in the same gene.

This information is important for several reasons. First, since mutation in the *db* gene can cause obesity in two species, these data suggest that the gene might enter into body weight control in other mammals, including humans. This possibility can be tested by analyzing cosegregation of RFLPs for *ifα* and *glut1* with an obese phenotype in human pedigrees. Suitable pedigrees from several places, including the Pima Indians and the Maracaibo Indians of Venezuela, are being collected. Second, these efforts have likely yielded another mutant allele of *db*. This will be helpful for the cloning of the *db* gene. The molecular nature of the *fa/fa* mutation will ultimately provide more information about its structure-function relationship. Finally, it will be quite useful in the long term to have a rat model for the *db* mutation, as most physiologic and neuroanatomic studies are more easily done on rats than on mice.

Molecular Mapping of the *tubby* and *fat* Mutations

Experiments have been initiated to clone the *fat* and *tubby* mutations. *fat* and *tub* are recessive, and both result in an obese phenotype that is somewhat less severe than that of *ob* and *db* mice. Mutant mice generally weigh ~60 grams. While little is known about the biologic features of these mutant animals, the implementation of the same techniques being used to clone *ob* and *db* can be applied to clone the *fat* and *tub* genes. Separate genetic crosses have been established between the *fat* and *tub* mice and *Mus castaneus*. These studies have confirmed the map positions of *fat* on chromosome 8 and *tub* on chromosome 7, laying the groundwork for the physical mapping of these mutations.

In summary, the new techniques of molecular genetics are being applied to the study of obesity and type II diabetes. These studies make use of mutant mice, with the goal of isolating the human homologues of the mutant mouse gene. The availability of cloned obesity genes will make possible molecular studies of the regulation of body weight in health and disease.

Dr. Friedman is also Associate Professor and Head of the Laboratory of Molecular Genetics at the Rockefeller University.

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MOLECULAR GENETICS OF THE MAJOR HISTOCOMPATIBILITY COMPLEX

Jan Geliebter, Ph.D, *Assistant Investigator*

Dr. Geliebter and his colleagues are studying the molecular mechanisms by which multigene families evolve as a unit, a process referred to as concerted evolution. The laboratory is focusing on the role of recombination in the concerted evolution of the murine major histocompatibility complex (MHC), as well as the function of its protein products.

Histocompatibility molecules are the proteins found on the cells of mammals that are responsible for graft rejection. The biological function of these molecules is to bind foreign antigens and present them to the immune system. Mice and humans have three to six different histocompatibility molecules, each of which can bind a limited number of antigens. To bind a large number of antigens and ensure the survival of the species, it is beneficial that many varieties of the histocompatibility molecules be present in the population.

The three murine histocompatibility loci, K, D, and L, collectively make up the H-2 region of the murine MHC. The H-2 genes are members of the much larger class I multigene family of the MHC (~30 genes), which also includes the Qa and Tla region genes. Alleles of each H-2 locus exhibit high sequence variation (diversity) and are very polymorphic in the population. Alleles of Qa and Tla region genes exhibit very little sequence diversity and are much less polymorphic than H-2 loci. Although the Qa and Tla region genes and their products share sequence homology and biochemical characteristics with the H-2 region genes and molecules, their biological role is unknown.

Genetic Recombination in Murine Cells

Mutant K^b genes have been detected in C57BL/6 mice (by skin grafting) at a high frequency of ~1 per 2,500. (Alleles of H-2 loci from the C57BL/6 strain are identified by a b superscript.) Sequence analyses have indicated that these mutant K^b genes contain clustered, multiple nucleotide alterations. The finding that other class I genes contain the identical sequences as substituted into the mutant K^b gene provides evidence that the mutant genes are generated by a recombination event between the K^b gene and other class I genes.

These recombinations, which result in the transfer of very small segments of DNA (<100 nucleotides) from donor genes to K^b, have been termed microrecombinations. The microrecombinant K^b genes are repetitive in nature; that is, the identical microrecombinant genes have been detected in independently arising mutant mice. It is thought that the products of many microrecombination events, accumulating in the K^b genes of a mouse population, result in the high sequence diversity observed among K^b alleles.

The emphasis of the research in Dr. Geliebter's laboratory is the analysis of the parameters and requirements of the microrecombination process. To gain further insight into this process, the laboratory is using *in vitro* engineered constructs to detect microrecombinant H-2 genes.

Dr. Geliebter's associates have constructed a fusion gene in which β-galactosidase sequences replace two cytoplasmic exons of the K^b gene. The fusion protein can be detected by staining for β-galactosidase activity, which is manifested as blue-colored cells. They have also site-directed two in-frame termination codons in the K^b gene at positions that undergo frequent microrecombinations. This prevents the expression of β-galactosidase. β-Galactosidase expression can be rescued by a mi-

crorecombination with a linked class I gene, $Q4$, which recombines away the termination codons. Thus microrecombinations can be scored as blue cells.

This microrecombination construct, once introduced into a variety of cell types, will help determine the microrecombination frequencies of different cells. Its introduction into transgenic mice will help determine microrecombination frequencies in germ cells. Data from previous studies indicate that microrecombinations occur in female germ cells. The laboratory also hopes to determine if microrecombinations occur in sperm cells as well, and at what frequency.

Some strains of mice may undergo microrecombinations more frequently than others. Placing this construct onto different genetic backgrounds may help to determine microrecombination frequencies in different mouse strains, and perhaps identify critical parameters in the microrecombination process. These studies will contribute to an understanding of the genetic processes that control the evolution and ultimately the function of the mammalian immune system.

Identification of a Diverse Qa Gene

While sequence diversity and polymorphism are the hallmark of H-2 genes, Qa and Tla region genes are characterized by sequence conservation among alleles and limited polymorphism. In fact, the lack of polymorphism among these genes has been suggested to preclude an immunological function for their products. Dr. Geliebter's laboratory has identified a Qa region gene whose sequence differs greatly between alleles of the C57BL/6 and C3H mice. The sequence diversity between the two alleles is manifested in both scattered and clustered nucleotide substitutions. The clustered substitutions are similar to those observed in the microrecombinations that diversify H-2 genes, and may reflect past microrecombination events with H-2 and other Qa region genes. These data may provide the first evidence that Qa genes can be recipients in the microrecombination process.

PCR (polymerase chain reaction) analysis has indicated that this Qa gene is transcribed in the thymus of at least some strains of mice. Dr. Geliebter's laboratory is currently engaged in an in-depth analysis of the transcription, translation, and cell surface expression of this gene and its product in order to ascertain its function. The gene is polymorphic in at least three strains of mice, and the analysis of other strains is under way. The diversity and polymorphism of the gene suggest an immunological func-

tion for its product, perhaps the first such function to be ascribed to a *Qa* gene.

Dr. Geliebter is also Assistant Professor and Head of Laboratory at the Rockefeller University.

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TRANSLATION

Raymond F. Gesteland, Ph.D., *Investigator*

Ribosome Hopping

Dr. Gesteland's laboratory has characterized the determinants in mRNA of *Escherichia coli* phage T4's gene *60* that allow ribosomes to bypass efficiently a 50-nucleotide coding gap, and studies are under way to explore this unusual biochemical mechanism. A genetic approach has been to look for bacterial mutants that restore hopping efficiency to mutants of gene *60* that are defective in the mRNA signals for hopping.

One mutation is a single amino acid substitution in ribosomal protein L9. This change restores hopping to constructs that are deficient in a stem structure located at the beginning of the gap sequences. Somehow the altered L9 can compensate for the lack of a particular structure in the mRNA. Little is known about the role that L9 normally plays in the ribosome. This new mutant should provide a useful tool for understanding both hopping and ribosomes.

A second mutant that restores hopping to many defects in the *60* mRNA (including altered amino acid sequences in the growing peptide chain) is, surprisingly, in the gene for lipoamide dehydrogenase. This defect in the TCA cycle results in an ATP deficit that elicits the stringent response and the synthesis of ppGpp, which somehow alters ribosome fidelity or kinetics to give the enhanced hopping phenotype.

The role of the 15–amino acid peptide is being studied by systematic mutagenesis. Sequential replacement of each residue with aspartic acid showed that many sites have a modest influence on hopping, and a few seem crucially important. Further constructs will guide biochemical experiments to elucidate the role of the peptide in hopping.

Messenger RNA Stability

A series of Shine-Dalgarno (SD) and initiation codon changes show that SD efficiency but not initiation codon strength is necessary for *lacZ* mRNA stability in *E. coli*. However, a point mutant in the fourth codon of *lacZ* is shown to affect translation initiation and, consequently, mRNA stability. The point mutant exerts its effect on translation initiation by creating an occluding secondary structure partly within the translation initiation region. The extent of the secondary structure and a putative stem-loop are investigated by gene fusion and site-directed mutagenesis. The secondary structure is shown to be contained within codons 1–23 of the *lacZ* transcript.

Readthrough

The *gag* and *pol* genes of Moloney murine leukemia virus (Mo-MuLV) are separated by an in-frame UAG codon. Translational readthrough of this stop codon allows synthesis of a *gag-pol* fusion polypeptide, the sole source of *pol* gene products. *In vitro* translation studies have shown that readthrough of the UAG codon is dependent on a 3′ pseudoknot that is separated from the UAG codon by an 8-nt purine-rich spacer.

Comparison of the Mo-MuLV pseudoknot sequence with other viruses that utilize stop codon readthrough shows the potential for pseudoknot formation in several of these viruses, a similar purine-rich sequence immediately 3′ to the UAG codon and a group of conserved nucleotides in the second loop of the pseudoknot. Several of these viral pseudoknot sequences have been tested and

shown to promote readthrough *in vitro*. Mutations within the Mo-MuLV spacer region have varied effects on readthrough.

These results imply a complex stimulator for readthrough that includes the downstream pseudoknot and local sequence context near the UAG codon. Focus is currently on the importance of the conserved nucleotides in the second loop of the pseudoknot and on pseudoknot structural analyses. Pseudoknot function in *Saccharomyces cerevisiae* is being studied to initiate a genetic approach to identifying cellular components that interact with the pseudoknot to promote readthrough.

DNA Sequencing Technology

Multiplex sequencing technology coupled with a transposon-based front end is being given a serious pilot test on human DNA to assess the efficiency and the problems of scale-up. Direct readout of chemiluminescent probes on the sequencing membranes with a CCD (charge-coupled device) camera is being developed with the goal of building an automated system. A sequence reading device has been developed and is being tested.

In collaboration with the Computer Science Department at the University of Utah, custom chips have been fabricated that do very fast parallel sequence comparisons. These are compatible with small computers and should greatly enhance the power of homology searches of the increasingly large DNA sequence database. Two technologies are being pushed for fast sequence comparisons of human populations.

Capillary technology is being developed for polymerase chain reaction (PCR)-based rapid assays of variants, and a multiplex approach that does direct comparison of raw sequences to look for differences is in early development.

Dr. Gesteland is also Professor of Human Genetics and of Biology at the University of Utah School of Medicine and Adjunct Professor of Bioengineering at the University of Utah.

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MOLECULAR BIOLOGY OF BLOOD COAGULATION

David Ginsburg, M.D., *Associate Investigator*

The research program of Dr. Ginsburg's laboratory focuses on the biology of the blood coagulation system and the molecular genetics of associated human diseases.

von Willebrand Factor and von Willebrand Disease

von Willebrand factor (vWF) is an adhesive plasma glycoprotein that plays a central role in hemostasis, both as the major mediator of platelet adhesion to the blood vessel wall and as the carrier for factor VIII (the antihemophilic factor). von Willebrand disease (vWD) is the most common inherited bleeding disorder in humans with prevalence estimated to be as high as 1–3% of the population. Over 20 distinct subtypes of vWD have been reported, all with subtle phenotypic differences.

Quantitative defects in plasma vWF, type I and type III, are the most common forms of vWD. Although gene deletions have been identified in rare cases, more detailed analysis has been difficult, given the large size of the gene. Dr. Ginsburg's laboratory has developed a novel polymerase chain reaction (PCR) approach using a panel of exonic DNA sequence polymorphisms that can be scored both at the DNA and mRNA level. Once a heterozygous polymorphism is identified in patient genomic DNA, PCR analysis of platelet vWF mRNA can detect decreased or absent expression from one or the other vWF allele. To date, such "null alleles" have been identified in 3 of 3 type III vWD families (recessive inheritance) but only 1 of 9 type I patients (dominant inheritance), suggesting a difference in the molecular mechanisms responsible for these two disorders.

The laboratory has recently begun to study an animal model for type I vWD in the RIIIS/J mouse, in collaboration with Dr. Richard Swank (Roswell Park). Surprisingly, early linkage data suggest that the murine disorder is not linked to the vWF gene. Defects outside of the vWF locus, potentially interfering with vWF biosynthesis or secretion, may also account for some or all of human type I vWD.

Type IIA vWD is associated with a selective loss of the large, most functionally active vWF multimers from plasma. Dr. Ginsburg and his colleagues have now identified seven distinct mutations in 9 of 11 unrelated type IIA vWD patients. All seven are clustered within a 123–amino acid segment of the vWF A2 domain, and one has been identified on three distinct genetic backgrounds. Analysis of these type IIA mutations by expression in heterologous cells has identified two distinct mechanisms responsible for the type IIA phenotype. In one group the mutation results in a block to intracellular transport at the level of the endoplasmic reticulum, more severely affecting larger vWF multimers. In the second group, normal intracellular processing is observed with the loss of large multimers occurring after secretion, presumably due to proteolysis in plasma. Interestingly, two type IIA vWD mutations have been identified in the same codon, one resulting in a secretory and the other a nonsecretory defect.

Type IIB vWD is characterized by markedly increased vWF binding to platelets, with the subsequent clearance of large multimers from plasma. A panel of four missense mutations, all clustered within a 35–amino acid segment of the vWF A1 domain, account for all 14 of the type IIB vWD patients studied to date. Insertion of the most common of these mutations into recombinant vWF confers markedly increased binding to platelets, accounting for the phenotype of this disorder. Again, several of these mutations have been shown to occur on distinct restriction fragment length polymorphism (RFLP) backgrounds, consistent with independent genetic origins and suggesting that only a limited number of mutations can produce this unique "gain-of-function" defect.

The information derived from these studies should provide the tools for precise genetic diagnosis and classification of vWD. Along with ongoing analysis of vWF structure and function, these studies may also lead to novel therapeutic strategies for the treatment of thromboembolic disease. This work has been supported in part by a grant from the National Institutes of Health.

Plasminogen Activator Inhibitor-1

Plasminogen activator inhibitor-1 (PAI-1), a member of the serine protease inhibitor (SERPIN) supergene family, serves as the major regulator for the plasminogen activators uPA (urokinase plasminogen activator) and tPA (tissue plasminogen activator). Abnormal plasma levels of PAI-1 may be associated with a variety of thromboembolic disorders in humans. In addition, PAI-1 has been hypothesized to play a critical role in diverse biologic processes, including ovulation, embryogenesis, tissue remodeling, and tumor metastasis. Current studies of PAI-1

in this laboratory are centered largely on structure/function analysis. Initial work has focused on the reactive center of PAI-1.

A large library of mutants has been constructed, containing all possible sequences at the P1 and P1′ residues. Analysis of 340 mutants to date has identified a requirement for either Arg or Lys at P1 for activity against uPA. The P1′ position appears to be tolerant of a wide range of amino acid substitutions, with the exception of Pro. Several of these novel reactive center variants demonstrate remarkable target specificity for uPA over tPA. Recently a new class of mutants has been identified with residues other than Lys or Arg at P1, which maintain selective inhibitory activity against tPA, with no detectable activity for uPA. In other studies, chimeric SERPIN molecules are being constructed, combining the amino terminus of α_1-antitrypsin or antithrombin III with the carboxyl terminus of PAI-1. By this approach, regions critical for the interaction of PAI-1 with vitronectin and heparin have been identified.

The laboratory has recently characterized the molecular defect in a patient with a hereditary bleeding disorder associated with low PAI-1 levels. She is homozygous for a 2-bp insertion in exon 4, resulting in complete loss of functional PAI-1 expression. Surprisingly, she demonstrates no obvious clinical abnormalities, aside from her moderate bleeding disorder. In addition to identifying the molecular basis for a new human bleeding disorder, these observations have important implications for the role of PAI-1 *in vivo*. Genetic analysis of additional patients with PAI-1 deficiency is in progress. This work has been supported in part by a grant from the National Institutes of Health.

Bone Marrow Transplantation

Currently the major obstacle to the more widespread use of bone marrow transplantation (BMT) to treat human disease is an often fatal complication called graft-versus-host disease (GVHD). GVHD does not occur in BMT between identical twins but is a frequent complication when bone marrow is used from a nonidentical but HLA-matched sibling. Thus one or more genetic loci outside of the HLA complex appear to be critical determinants of GVHD. Dr. Ginsburg has recently begun a new research program, attempting to identify these loci by a "genome scan" analysis of highly polymorphic microsatellite markers. If this effort is successful, the identified markers will find immediate application in the refinement of the BMT-matching procedure. In addition, identification of the actual genetic loci should open important avenues for future research.

Dr. Ginsburg is also Associate Professor of Internal Medicine and Human Genetics at the University of Michigan Medical School.

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MOLECULAR BASIS OF X-LINKED DISORDERS

Jane M. Gitschier, Ph.D., *Assistant Investigator*

This laboratory is interested in a number of aspects of X-linked disorders, such as uncovering the genes responsible for some of these and finding the mutations that underlie them. In addition, research is aimed at improving genetic prediction and therapy for these diseases. Research in the past year has led to progress in a number of projects.

Isolation of Candidate Disease Genes in Xq28

Xq28 is the terminal band of the long arm of the human X chromosome. It is thought to be an extremely gene-rich region, and at least 20 inherited diseases are known to map here. This laboratory is in the process of isolating genes in Xq28 and determining whether mutations in any of them are associated with a particular inherited disorder.

To date, six genes have been isolated, the most intriguing of which is the *MPP-1* gene. *MPP-1* was found to code for a membrane protein previously described in erythrocytes, but probably expressed in all cells. This protein, p55, is heavily palmitoylated and contains domains homologous to SH3 (src-homology domain 3) and to the enzyme guanylate kinase. Overall, its sequence is reminiscent of the derived amino acid sequence of the *Drosophila* tumor-suppressor gene *lethal discs large (dlg)*. Mutations in this gene lead to overgrowth of the epithelial cells in imaginal discs.

It is tempting to speculate that mutations in MPP-1 could lead to a genetic disease, and several candidates come to mind. One is dyskeratosis congenita, an Xq28-linked disorder characterized by leukoplakia of the oral mucosa, nail dystrophy, continuous lacrimation, and a tendency for malignancy. Mutations in the p55 gene might also lead to one of the muscle disorders mapping to Xq28, since muscle cells may be particularly vulnerable to mutations that affect membrane integrity.

The laboratory is in the process of screening patient samples for mutations in the *MPP-1* gene, as well as in other genes. Samples from more than 70 patients with Xq28-linked disorders have been collected and immortalized. These are being assayed for abnormalities in DNA, RNA, and protein.

Identification of Mutations in the V2 Vasopressin Receptor Gene

Nephrogenic diabetes insipidus (NDI) is one of the inherited diseases that map to Xq28. In patients with this disorder, water retention by the kidney is not responsive to the antidiuretic hormone vasopressin. Recently isolation of the gene for the kidney-specific vasopressin receptor was reported. Because it was discovered to map to Xq27-28, it is a likely candidate for the NDI gene. Dr. Gitschier's laboratory made use of the published sequence to design primers for RT-PCR (reverse-transcriptase/polymerase chain reaction) of RNA prepared from the immortalized NDI cell lines. Independent mutations were found in five of six unrelated patients tested. These mutations include one nonsense mutation, one frameshift, three missense mutations affecting conserved amino acids, and remarkably, in one patient, an additional 12-bp deletion. These findings strongly support the hypothesis that the vasopressin receptor is defective in NDI.

Isolation of a Candidate Gene for Menkes Syndrome

Menkes syndrome is an X-linked disorder of copper metabolism resulting in progressive neurological degeneration and death in early childhood. The underlying etiology is unknown, and there is no predictably effective treatment. Genetic linkage studies point to Xq13 as the location of the Menkes gene. Although Menkes disease is almost exclusively found in males, a female patient has been identified with a balanced X:autosomal translocation, the breakpoint mapping to Xq13. It is hypothesized that the breakpoint of the translocated chromosome disrupts the Menkes gene.

In collaboration with Dr. Seymour Packman (University of California, San Francisco), this laboratory has undertaken to isolate the gene responsible for Menkes disease. DNA probes from the Xq13 region were obtained and hybridized to blots of pulsed-field gels of normal and translocated DNA to localize the breakpoint. Yeast artificial chromosomes (YACs) that span the breakpoint were then isolated with the closest flanking probes, and the breakpoint position was refined to within 2 kb. These genomic sequences were used to isolate cDNA sequences by several approaches, including direct YAC screening and exon trapping.

An excellent candidate cDNA has been obtained. The sequence for this large cDNA shows homology to a cadmium-transport protein from *Staphylococcus aureus* and to the class of cation-transporting ATPases. The occurrence of aberrations in both the

DNA and RNA of several unrelated Menkes patients strongly supports the hypothesis that mutations in this gene lead to the Menkes syndrome.

Analysis and Inactivation of the Murine Factor VIII Gene

Dr. Gitschier's laboratory has a long-standing interest in the molecular genetics of hemophilia A. This X-linked bleeding disorder results from mutations in the gene coding for coagulation factor VIII, a large and unstable clotting cofactor.

Recent efforts in this laboratory have been directed toward making a mouse model for hemophilia for future gene therapy experimentation. Portions of the murine genomic sequences coding for factor VIII have been isolated and mutagenized by insertion of a neomycin-resistance gene. These sequences will be used to disrupt the native murine gene in embryonic stem cells by homologous recombination. In theory, a factor VIII–deficient mouse could be derived from the targeted cells.

In a complementary investigation, the murine cDNA has been isolated and sequenced. The predicted protein shows almost 90% amino acid identity with the human protein in the A and C domains, portions of the molecule that are required for activity. In contrast, the sequences of the B domains, which are dispensable, are only 50% identical. Sequence of the murine cDNA, as well as factor VIII cDNAs from other species, should be valuable for interpretation of the effects of missense mutations on the structure or function of factor VIII.

The hemophilia research described above was

supported through a grant from the National Institutes of Health.

Dr. Gitschier is also Associate Professor of Medicine at the University of California, San Francisco.

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CHROMOSOME ORGANIZATION AND GENE FUNCTION IN *DROSOPHILA*

STEVEN HENIKOFF, PH.D., *Investigator*

Position-Effect Variegation

When chromosome breaks cause rearrangements in the germline of *Drosophila*, position effects are sometimes seen on genes close to the resulting breakpoints. The most curious of these position effects are those in which heterochromatin (the compacted pericentromeric regions) and euchromatin are juxtaposed. Euchromatic genes next to such breakpoints often show variegated expression, indicating inactivation in some cells but not in others. This is a long-range phenomenon, often affecting the expression of genes that are on the order of a

megabase from the breakpoint. Although position-effect variegation has been studied for several decades, a mechanistic understanding has proved elusive.

One of the most fascinating aspects of this phenomenon is that inactivation is often clonal, as if a decision made early in development is stably transmitted through several cell generations. However, a problem in trying to study clonal inactivation is that the phenotype is only seen after cell division is complete. Work in Dr. Henikoff's laboratory has now led to the isolation of a mutation that causes position-

effect variegation on a *lacZ* fusion gene expressed in developing tissue, making possible the investigation of this phenomenon in dividing cells.

An aspect of the phenomenon that is becoming clearer is the dominance of some variegation mutations over wild type. Although variegation is always recessive for nearly all genes, variegated mutations of the *brown* gene are always dominant. That is, when the *brown* gene is juxtaposed to heterochromatin, variable inactivation is seen for the copies in both cis and trans, whereas other genes are only affected in cis. Investigations in Dr. Henikoff's laboratory have revealed that a necessary component of "trans-inactivation" of *brown* is somatic pairing of homologues. It is proposed that trans-inactivation results from direct contact between protein components responsible for the heterochromatic state of the cis copy of the gene and other components associated with the trans copy.

In support of this model, the trans-inactivation phenomenon has been reproduced at sites of transposons carrying the *brown* gene, but only for paired copies of the gene. By taking advantage of the ability to make small deletions *in vivo* in the transposon system, it has been found that disruption of chromosome pairing in the immediate vicinity of the gene reduces trans-inactivation, as if a cis-acting component of trans-inactivation is very close to or within the *brown* gene itself. Current efforts are aimed at mapping the precise sequence location of this cis-acting component, which appears to be within ~1 kb of the 5′ end of the coding region.

A Somatically Unstable Chromosome

Dr. Debora Wines has discovered a chromosome that shows an extreme instability in somatic cells. This serendipitous finding was made during a screen for position-effect variegation mutations: the unstable chromosome caused gene markers carried on it to appear variegated. This chromosome derives from a translocation involving ~20% of the left arm of chromosome 3 and a centromere from another chromosome. The unstable chromosome is intermediate in size among wild-type and rearranged linear *Drosophila* chromosomes, all of which are known to be quite stable. The instability results from random nondisjunction of sister chromatids during development, leading to clones and single cells that have either gained an extra copy of the chromosome or have lost it entirely. An intriguing possibility is that the defect results from a position effect on the centromere, suggested by the observation that genetic elements that modify position-effect variegation modify the degree of instability. This unique chromosome may provide a tool for the genetic

and molecular dissection of a higher eukaryotic centromere.

Homology Searching and Scoring Matrices Based on Protein Blocks

A system has been developed for finding and assembling the most highly conserved regions of related proteins for database searching. This involves automation of an algorithm for sensitive detection of multiple local alignments followed by conversion to "blocks" of aligned protein segments and assembly of the best set of blocks. The automated system was applied successively to more than 500 groups of related proteins, yielding a database of about 2,000 blocks that could itself be searched for distant relationships. The practical use of blocks has been demonstrated by the detection of previously unknown relationships and the evaluation of relationships proposed by others.

A special feature of using blocks in searching is that more than one block from a group can be detected independently, allowing inferences of homology to be made with confidence based on the low probability of detecting multiple blocks by chance. Among the relationships detected with this approach were new vertebrate and bacterial members of the Tc1 family of transposons, previously thought to be limited to invertebrates.

Since the system for finding and assembling blocks is fully automated, the database is kept up to date and made available over Internet. In addition, an electronic mail server is maintained so that the current database can be searched by other researchers without requiring special software or hardware.

The database of blocks was also used to derive amino acid substitution matrices for scoring protein alignments. This is a different approach from that used to derive the widely used matrices based on the Dayhoff model of evolutionary rates. This approach led to marked general improvements in alignments and in searches for homology with representatives from hundreds of different protein families.

Dr. Henikoff is also Member of the Division of Basic Sciences of the Fred Hutchinson Cancer Research Center, Seattle, and Affiliate Associate Professor in the Department of Genetics at the University of Washington, Seattle.

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PROTEIN-MEDIATED PROTEIN FOLDING

ARTHUR L. HORWICH, M.D., *Associate Investigator*

Chaperonins

A major portion of the research in Dr. Horwich's laboratory is aimed at understanding how proteins acquire their functional structures in the living cell. Newly translated proteins emerging from ribosomes and proteins translocated through membranes must be folded into the correct tertiary structures to achieve biological activity. For many years this was believed to be a spontaneous process, but recent studies indicate that special protein components called molecular chaperones mediate folding *in vivo*. Dr. Horwich's laboratory uncovered such a component in mitochondria: heat-shock protein 60 (hsp60). It was the component affected in a temperature-sensitive lethal mutant of yeast in which proteins imported into the mitochondrial matrix failed to be folded and assembled into active forms. The misfolded proteins were found as insoluble aggregates.

Residing in the mitochondrial matrix as a homo-oligomeric 14mer complex, hsp60 is composed of two stacked rings, each containing seven radially arranged 60-kDa monomers. It binds newly imported mitochondrial proteins in unfolded forms and mediates folding in a process requiring ATP and a second cooperating component. It shares 60% of its amino acids and the same quaternary structure with both a protein of the bacterial cytoplasm, groEL, and the RUBISCO-binding protein of chloroplasts. The three related components, termed chaperonins, are probably evolutionarily related through events of endosymbiosis from which mitochondria and chloroplasts emerged.

Chaperonin Mechanism

In vitro studies with groEL have reconstituted the native active form of several monomeric proteins diluted from denaturant. In studies carried out in collaboration with Dr. Ulrich Hartl and his co-workers, groEL-mediated folding was dissected into two steps: 1) binding of an unfolded polypeptide, with a stoichiometry of one or two molecules per groEL 14mer complex, with stabilization in the conformation of an early-folding intermediate called "molten globule," containing a native-like secondary structure, a nonorganized tertiary structure, and solvent-accessible hydrophobic residues; and 2) folding, mediated upon addition of MgATP and the cooperating component groES, a seven-member ring composed of 10-kDa monomers, which binds with a stoichiometry of one groES ring per groEL 14mer. Folding is associated with ATP hydrolysis (~100 ATP/monomer), presumed to be expended in rearrangement of the groEL structure. The presence of groES appeared to couple the substrate polypeptide to groEL, potentially enabling its stepwise folding and release.

Chaperonin-like Heat-Shock Protein in Thermophilic Archaebacteria and a Relative in the Eukaryotic Cytosol

The mechanism of chaperonin-mediated folding may not be confined to the compartments in which groEL, hsp60, and RUBISCO-binding protein are localized. The major heat-shock protein of thermophilic archaebacteria was discovered to exhibit a similar quaternary structure and functional features,

and its primary structure was found to be 40% identical to that of a protein of the eukaryotic cytosol, t-complex polypeptide-1 (TCP1).

Thermophilic factor 55 (TF55) has quaternary structure of a chaperonin. TF55 was purified from *Sulfolobus shibatae*, a thermophilic archaebacterium that normally grows at 75°C. TF55 is a major 55-kDa polypeptide in *S. shibatae* at this temperature, but after a shift to the near-lethal temperature 88°C, it is virtually the only protein synthesized. Its production at 88°C correlates with acquisition of thermotolerance: cells incubated first at 88°C can survive subsequent shift to the otherwise lethal temperature of 95°C.

In sucrose density-gradient sedimentation, TF55 migrated as a 20S homo-oligomer, a behavior reminiscent of the chaperonins. In scanning transmission electron microscopy, carried out in collaboration with Dr. Joseph Wall, a double-ring structure was observed, resembling that of the chaperonins. Instead of seven-member rings, however, TF55 complex contained nine-member rings.

Purified TF55 complex has biochemical properties of a molecular chaperone. In collaboration with Dr. Hartl, Dr. Horwich and his colleagues observed that TF55 complex binds mesophilic proteins as they become thermally unfolded at 56°C and 70°C but does not bind thermophilic proteins that remain in native form at these temperatures. Like the chaperonins, TF55 complex exhibits ATPase activity, with the rate of hydrolysis at 75°C equivalent to that of groEL at 37°C.

Predicted amino acid sequence of TF55 is 40% identical to that of a eukaryotic protein, TCP1. TCP1 was originally identified as an abundant 58-kDa protein in mouse testis that mapped to the t-locus. A role in male-specific transmission ratio distortion was proposed, but the protein subsequently proved to be ubiquitous in mammalian tissues and to be present also in *Drosophila* and *Saccharomyces cerevisiae*. In yeast the gene was shown to be essential, and a cold-sensitive mutant exhibited an abnormal-appearing mitotic spindle.

TCP1 complex is a molecular chaperone in tubulin biogenesis. In collaboration with Dr. Himan Sternlicht and his co-workers, a role for TCP1 as a chaperone in the biogenesis of tubulin was assessed. Dr. Sternlicht's group had previously observed that both α- and β-tubulin subunits translated in reticulocyte lysate were transiently associated with a 900-kDa complex prior to appearance in monomer or α-β heterodimer forms. Release of newly translated tubulin subunits from the 900-kDa complex could be blocked by depletion of ATP with apyrase. Tubu-

lin subunits associated with the complex were found to be exquisitely protease sensitive, compared with a high degree of protease resistance of monomer or heterodimer forms corresponding to that of native tubulin. This suggested that while bound by the complex, tubulin was present in an unfolded conformation, but in the presence of MgATP it was folded to a native conformation.

Analysis of the purified 900-kDa complex revealed a collective of at least seven species of apparent molecular size 55–60 kDa. One of these species, a 58-kDa protein, gave a signal on immunoblot analysis with a monoclonal antibody against mouse TCP1. The TCP1-containing complex thus differs from the chaperonins insofar as it appears to be a hetero-oligomer. How general its role may be in mediating protein folding in the eukaryotic cytosol remains to be determined by *in vivo* studies of both a temperature-sensitive mutant affecting yeast TCP1, isolated in Dr. Horwich's laboratory, and mutants affecting the other members of the complex.

Dr. Horwich is also Associate Professor of Genetics and of Pediatrics at Yale University School of Medicine.

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TRANSCRIPTIONAL CONTROL MECHANISMS COMMON TO B CELLS AND MYELOID CELLS

Thomas R. Kadesch, Ph.D., *Associate Investigator*

During the past several years, Dr. Kadesch's laboratory has studied the immunoglobulin heavy-chain (IgH) enhancer as a cell type–specific transcriptional control element. The goal of this work is not simply to understand transcriptional control in B cells but to establish paradigms that may apply to other cell types and other gene systems. Results obtained over the past year have demonstrated that B cells and myeloid cells utilize similar mechanisms to regulate their differentiation programs and to control expression of their genes.

Immunoglobulin Gene Transcription: the Heavy-Chain Enhancer

The activity of the IgH enhancer is restricted to B cells, and this is dictated by both positive and negative control mechanisms. Positive control stems from B cell–specific proteins, such as those represented by the helix-loop-helix family of DNA-binding proteins, and from DNA-binding proteins ubiquitously expressed. Negative control, functioning in non–B cells, assures that the activity of the ubiquitously expressed proteins is repressed.

Previous work from Dr. Kadesch's laboratory indicated that repression of the enhancer in non–B cells is mediated through a particular site, designated μE5. That the presence of the μE5 site represses enhancer activity can be measured through a neighboring site, μE3. The μE3 site binds the ubiquitous transcription factor TFE3, which only in the absence of the μE5 site is sufficient to activate the enhancer in non–B cells. The hypothesis is that in non–B cells the μE5 site binds a repressor that inhibits the activity of proteins acting through that site. In B cells the μE5 site functions in an opposite manner to stimulate enhancer activity by binding its own positive transcription factor. This factor is related or identical to the protein E2-5. When artificially overexpressed in non–B cells, E2-5 can displace the repressor and activate the enhancer. This raises the possibility that the activator and repressor exist in the same cell and that enhancer activity (on or off) is dictated by the ultimate outcome of their competitive μE5 binding.

In an effort to understand enhancer regulation mediated through the μE5 site, a clone representing a candidate repressor protein has been isolated from a HeLa cell cDNA library. Isolated through its ability to bind the μE5 site, this cDNA encodes a zinc finger protein ($[C_2H_2]_3$ type), and the protein was accord-ingly designated Zeb (for zinc finger E5-binding protein). Zeb possesses two distinct regions containing zinc finger motifs. Each of these motifs is sufficient for DNA binding, and each apparently has the same DNA-binding specificity. The discovery of Zeb is encouraging, because all the μE5-binding proteins previously identified (including E2-5) belong to a distinct family of transcription factors that bind DNA through a basic-helix-loop-helix (bHLH) motif. The presence of the zinc fingers suggests that ZEB's mode of action might be different.

The μE5 site of the IgH enhancer is a member of a family of related sites, collectively referred to as E boxes. These are defined by the DNA sequence CANNTG and are found associated with a variety of genes expressed in cell type–specific manners. They generally bind bHLH proteins *in vitro*, such as E2-5, but only those of a subset bind Zeb. When these various E boxes are substituted for the μE5 site in the IgH enhancer, only those that bind Zeb *in vitro* are able to confer repression *in vivo*. This is the strongest argument that Zeb actually encodes the repressor; additional support for that contention is still being pursued.

The IgH enhancer is subject to an additional type of negative regulation. In this case inhibition is mediated by a class of helix-loop-helix proteins that lack the ability to bind DNA and, by interacting with positive-acting bHLH proteins, render them also unable to bind DNA. The first reported member of this class of proteins is the protein Id. Id is down-regulated during development in a number of different cell lines, and it has been suggested that Id may be a general inhibitor of the differentiated state, by blocking the action of bHLH proteins specifically required for development (such as MyoD). Given that bHLH proteins play a role in activating the IgH enhancer, it was reasoned that Id may function similarly during B lymphoid development. In this scenario, Id would be expressed at high levels only in immature B cells, in which the enhancer has not yet been activated.

Results from Dr. Kadesch's laboratory suggest that Id is indeed involved in repressing the IgH enhancer during early stages of B lymphoid development. In collaboration with Drs. Stephen Desiderio (HHMI, Johns Hopkins University) and Robert Benezra (Memorial Sloan-Kettering Cancer Center), the laboratory demonstrated that Id is not expressed in most B lymphoid cell lines, including a large array of early

pro–B cells that have not yet undergone IgH gene rearrangement. All of these cell lines express sterile μ transcripts, indicating that the IgH enhancer is active. Only two cell lines were found to express Id, and these do not express sterile μ transcripts. Hence an inverse correlation exists between Id expression and enhancer activity. What remains to be determined, however, is whether ectopic expression of Id in such cells can block the differentiation process.

A Role for Helix-Loop-Helix Proteins in Myeloid Differentiation

To explore the role of helix-loop-helix proteins in other hematopoietic cell types, Dr. Kadesch and his colleagues used the cell line 32D. These cells are interleukin-3 (IL-3)-dependent myeloblasts that, on exposure to granulocyte colony-stimulating factor (GCSF), differentiate to mature neutrophilic granulocytes. In collaboration with Dr. Giovani Rovera (Wistar Institute), the laboratory showed that these cells required the activity of bHLH proteins to differentiate. This was based on the following observations. 1) Id mRNA levels in these cells drop upon GCSF treatment (to 10% pretreatment levels) and then rise (to 80–100% pretreatment levels). 2) Proteins capable of binding an E-box probe appear in 32DCl3 nuclear extracts only after Id mRNA levels drop, and then this DNA-binding activity disappears after Id mRNA levels subsequently rise. 3) Forced expression of Id in these cells (using an Id cDNA) abolishes the appearance of the E-box–binding activity, and the cells do not differentiate; they undergo apparent apoptosis upon treatment with GCSF.

Although the late up-regulation of Id in 32D cells clearly distinguishes them from B cells (Id is stably shut off during B cell development), it is clear that the actions of bHLH proteins are required in both cell types. Questions concerning the identity of the induced E-box–binding activity in 32D cells and the consequences of Id up-regulation later in development are currently being addressed. The work on 32D cells was carried out with funds from Dr. Rovera's research grants and was not supported directly by HHMI.

Dr. Kadesch is also Associate Professor of Human Genetics at the University of Pennsylvania School of Medicine.

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GENETIC DISORDERS OF ERYTHROPOIESIS

YUET WAI KAN, M.D., *Investigator*

Dr. Kan's laboratory has been investigating the molecular basis of genetic disorders affecting the red cells, with primary emphasis on sickle cell anemia, thalassemia, and hereditary hemolytic anemia due to membrane defects. The pathophysiological mechanisms of these diseases are being studied, newer methods of detecting the defects designed, and approaches for treatment investigated.

Detection of Sickle Cell Anemia and Thalassemia

An objective of this laboratory is to devise simple, rapid, nonradioactive tests for the hemoglobinopathies and thalassemia, which are prevalent in many developing countries. Methods that have been used include denaturing gradient gel electrophoresis to detect unknown mutations in the globin gene, and

fluorescent primers to identify point mutations such as sickle cell anemia and other hemoglobinopathies. Presently under investigation is the reverse dot-blot method, in which oligonucleotides are immobilized to filters and the patients' amplified DNAs are hybridized to them. Mutations in 35 different β-thalassemia lesions as well as those in the hemoglobinopathies such as S, C, and E could be detected rapidly in this manner.

Panels of filters are being designed for different regions of the world where β-thalassemia is common, including Asia, Africa, and the Mediterranean. Dr. Kan and his colleagues have collaborated with investigators in Sicily to initiate testing programs using this approach. Also communications are being set up with 10 regional centers in the Guangdong province of China to institute testing programs in a region of 60 million population and a carrier rate of ~3%.

Control of Globin Gene Expression

An important discovery made in the study of the control of globin gene expression is the identification of the functional importance of four hypersensitive sites (HS 1–4) upstream of the globin gene clusters. This region, called the locus control region (LCR), is believed to be responsible both for the high-level expression of globin genes in the erythroid cells and for the developmental switches from embryonic to fetal to adult globin expression. The mechanism may be mediated through a series of DNA-protein interactions between the HS of the LCR and the globin gene promoters. To investigate these physiologically important interactions, Dr. Kan's laboratory has utilized the *in vivo* footprinting technique to locate them in erythroid cells.

Critical protein-binding motifs have been identified in the hypersensitive sites, including NFE2/AP1, GT/CAC, and GATA-1 sequences. Transfection experiments demonstrated that the NFE2/AP1 sequences are critical for the action of the HS, as mutations or deletions abolish the function of the site. The GT and GATA sequences appear to be complementary but not absolutely essential for the enhancing activity. In cell lines that synthesize different types of globin, there was no discernible change in DNA-protein interactions in the different hypersensitive sites of the LCR, but the promoter region of the globin genes became footprinted in its TATA, CAAT, and CAC sequences when those genes became active.

The utility of these protein-binding motifs is being tested in gene transfer experiments. Because of the critical importance of the NFE2/AP1 sequences, they were inserted in a retrovirus vector that also carries the β-globin gene. The NFE2/AP1 doubles retrovirally introduced β-globin gene expression in erythroleukemia cells. Because the GT and GATA sequences were observed to be complementary in activity, they are being added to the vectors that carry the NFE2/AP1 site in order to seek further enhancement of globin gene expression.

Another way to control the diseases in sickle cell anemia and β-thalassemia is to increase γ-globin gene expression. Dr. Kan's laboratory has studied the effect of butyrate on globin gene expression, in collaboration with Dr. Susan Perrine (Children's Hospital Oakland Research Institute). Dr. Perrine found that butyrate increased fetal hemoglobin production in tissue culture cells. In the fetal lamb model, the infusion of butyrate suppresses the switch from fetal to adult hemoglobin expression. Preliminary experiments showed that butyrates also enhance γ-globin gene expression in patients with thalassemia. The mechanism of induction of fetal hemoglobin expression by butyrate is being investigated.

Red Cell Membrane Defects

Dr. Kan's laboratory has investigated the molecular basis of hemolytic anemias associated with elliptocytosis and spherocytosis. Previous studies had demonstrated that defects in protein 4.1, spectrin, and glycophorin could result in this group of disorders. Protein 4.1 is present not only in the erythroid cell but in all tissues, and many isoforms are produced from a single gene by alternate splicing. A family with severe hemolytic anemia due to the complete absence of protein 4.1 unexpectedly revealed no other systemic defects. DNA analysis disclosed a rearrangement of the gene in the region of the initiation codon of the red cell protein 4.1. However, another upstream AUG is utilized for the synthesis of nonerythroid protein 4.1. Therefore the rearrangement of the downstream AUG region does not affect the production of the nonerythroid protein 4.1. The patients who have complete absence of erythroid protein 4.1 have no defects in other tissues, as the nonerythroid protein 4.1 continued to be produced normally.

Dr. Kan is also Professor of Laboratory Medicine and Medicine and the Louis K. Diamond Professor of Hematology at the University of California, San Francisco, and Honorary Professor at the University of Hong Kong.

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STRUCTURAL AND FUNCTIONAL ORGANIZATION OF HOMEOTIC LOCI

Thomas C. Kaufman, Ph.D., *Investigator*

Dr. Kaufman's laboratory continues its efforts to understand the regulation and role of the homeotic genes during the developmental process in *Drosophila melanogaster*. Efforts focus on three members of the *Antennapedia complex* that control segmental identity in the head and anterior thorax of the embryo and adult: *labial* (*lab*), *proboscipedia* (*pb*), and *Sex combs reduced* (*Scr*). Each was chosen for its domain of expression and accessibility to molecular dissection.

Additionally, a set of chimeric genes has been constructed that allows ectopic expression of the protein products of these three loci as well as *Deformed* (*Dfd*), *Antennapedia* (*Antp*), and *Ultrabithorax* (*Ubx*). One set of chimeras utilizes the heat-shock–inducible promoter hsp70.

These chimeras have been used to drive ubiquitous expression of the homeotic protein products. Dr. Josef Heuer has studied the effects of this expression on the development of the peripheral nervous system (PNS) of the embryo. He has found that each of the proteins has specific effects on the developing PNS, and homeotic proteins normally expressed anteriorly (e.g., in the head) have effects on the PNS of the abdomen, despite showing no influence on the identity of the epidermally derived cuticle of these same posterior segments.

Inherent limitations of the heat-shock system have led to the utilization of the bipartite system originally developed by Dr. Andrea Brand in Dr. Norbert Perrimon's group (HHMI, Harvard Medical School). The system utilizes constructs encoding the yeast GAL4 transcription factor, which can be placed downstream of any promoter-enhancer combination driving expression in specific and unique patterns. The second component involves the UAS target sequences of GAL4, which are now cloned upstream of cDNA clones for all of the above-mentioned homeotics. Dr. Barbara Hamilton has recovered and characterized fly stocks containing each of the UAS-homeotic constructs. These have been combined with two GAL4 lines that are specifically expressed in the developing mesoderm and the nervous system, respectively.

Dr. Hamilton has been able to drive expression of all homeotics ectopically and specifically in these tissues. This ectopic expression causes either late embryonic or larval lethality but is not associated with any striking abnormalities in morphogenesis. Any effects on the normal expression patterns of the resident homeotic loci in either the mesoderm or central nervous system (CNS) have yet to be observed. These studies are currently extending to an analysis of GAL4 constructs driven by the *lab* and *pb* promoters.

The *Sex combs reduced* locus

Scr establishes the identity of the labial and prothoracic segments and is expressed primarily in the ectoderm. Dr. Kaufman and his colleagues are using *Scr* clones to investigate the molecular basis of *Scr* regulation through the identification of positive and negative cis-acting control elements. To localize these elements, the 80-kb upstream regulatory region and transcribed portions of the gene have been divided into 18 overlapping subfragments. These were subcloned into P-element vectors containing either an *hsp70-lacZ* or *Scr-lacZ* chimera, and fusion gene expression was assayed in transformant embryos.

The largest of the *Scr-lacZ* fusions contains 2.3 kb of upstream sequence as well as the first exon and intron of the gene. This construct is expressed over a domain that includes the intercalary segment anteriorly and the third thoracic segment posteriorly. Addition of a specific fragment normally located more than 30 kb upstream serves to truncate this ectopic pattern to one more reflective of the normal *Scr* pattern.

In addition, several DNA fragments can be shown to direct patterns of fusion gene expression, depending on whether they are upstream of the heterologous *hsp70* or homologous *Scr* promoter. This suggests that there may be promoter-specific interactions between regulatory elements in this interval.

Genetic evidence suggests that *Scr* regulation during larval development is sensitive to homologue pairing. Three DNA fragments from the *Scr* regulatory interval have been isolated that, when in a P-element vector containing a *white* minigene, induce the mosaic repression of *white* expression in the eye. This repression is either enhanced or only observed when transformant lines are made homozygous. This result suggests that these DNA fragments contain pairing-sensitive negative regulatory elements and likely house the genomic sequences that are responsible for the observed "transvection" at the *Scr* locus. The mosaic *white* expression appears to be set during the first larval instar and maintained until the eye is pigmented in the pupal stage.

Moreover, the degree of mosaicism depends on the dosage of *Polycomb*-group and *trithorax*-group gene products. This interaction has been shown to depend on the paired state of the transgene. Therefore it would appear that the pairing-sensitive regulatory elements present in these DNA fragments interact with genes necessary for the maintenance of the normal patterns of homeotic gene expression and that the identified fragments are likely relevant to normal *Scr* regulation. (This work is supported by a grant from the National Institutes of Health.)

Dr. Maureen Gorman has augmented these analyses by constructing a set of *Scr* minigenes. These behave like the reporter constructs described above, in that they exhibit ectopic patterns of expression. It was surprising, therefore, when stable transformed lines were recovered, since expression of the *Scr* protein product outside its normal domain should have deleterious effects. Surviving transgenic adults, however, do show homeotic transformations of the thorax and head capsule that mimic the phenotype of animals bearing heat-shock *Scr* constructs subjected to specific heat-shock regimes.

The *proboscipedia* locus

During embryogenesis, *pb* is expressed in cells of the mesoderm, maxillary lobes, labial lobes, and ventral nerve cord. In imaginal tissues, *pb* accumulates in cells of the CNS and the labial discs. Null mutations of *pb* that result in the transformation of labial palps to first thoracic legs were rescued with a *pb* minigene constructed from genomic sequences that lie within the genetically defined limits of the locus. Reporter gene constructs containing the first exon and upstream regions from the minigene do not accumulate β-galactosidase in a *pb* pattern. However, when a 1.6-kb region from intron 2 is placed within the 7.3 bp of upstream sequence, the vector is competent to direct β-galactosidase in a *pb* pattern in both embryos and third instar larvae.

The expression patterns of a series of minigene deletion constructs were examined to further the dissection of *pb* regulatory sequences. Two internal deletion lines were made that remove sequences within the intron 2 region. Additionally two 5' deletion lines were created. One removes sequences upstream of 1.6 kb; a second deletes all sequences except 90 bp upstream of the transcription start site.

The two 5' deletion minigene lines rescue the *pb* homeotic phenotype and accumulate wild-type levels of *pb* protein in the labial discs. Therefore sequences upstream of −90 bp are apparently not needed to activate *pb* in the correct spatial pattern in this imaginal tissue. However, both 5' deletion minigenes additionally exhibit ectopic *pb* accumulation in the eye-antennal disc, suggesting that the sequences deleted are important for repressing *pb* in regions outside the wild-type domain. The −90-bp 5' deletion line additionally directs *pb* protein ectopically in the leg discs. Therefore the region between −1.6 kb and −90 bp seems to be required to repress *pb* in the legs.

In contrast to the 5' deletion constructs, neither of the intronic deletions is capable of completely rescuing the *pb* mutant phenotype. The region in intron 2 thus appears to be essential for *pb* function, and removing sequences within it results in aberrant adult mouthparts characteristic of *pb* hypomorphic or amorphic mutants. These sequences do not direct *lacZ* in a *pb* pattern in combination with a heterologous promoter (hsp70) but are capable of regulating *lacZ* in a *pb* pattern when located in a reporter gene containing the *pb* promoter. Thus the conserved region appears to require the *pb* promoter to function properly.

At present the simplest model predicts that the second intron enhancer region coupled with the *pb* promoter is capable of directing *pb* protein in a broad region both anterior and posterior to the wild-type domain. Refinement into the normal *pb* pattern is achieved by the added action of the upstream repressor elements defined above. (This work is supported by a grant from the National Institutes of Health.)

The *labial* locus

The cis-acting elements of the *lab* locus have been defined in a similar manner to that described for *pb* and *Scr*. The protein products of this gene are normally accumulated in the ectoderm of the intercalary segment and dorsal ridge, the tritocerebral ganglion, and the endoderm of the anterior and posterior midgut. A series of reporter and minigene constructs have demonstrated that each of these spatial domains of expression is separately controlled by a unique set of cis-acting regulatory elements. Furthermore these elements interact with a distinctive set of trans-acting loci.

Initiation of expression in the intercalary segment requires only 750 bp of upstream sequence and the *lab* promoter. This expression is triggered by the action of the *buttonhead* and *empty spiracles* genes. Maintenance of *lab* expression, however, requires the presence of *lab* protein, i.e., *lab* is autogenously regulated. This feedback loop also requires an additional 3.0-kb fragment of upstream DNA and only operates in the ectoderm of the intercalary seg-

ment. The ectoderm of the dorsal ridge is controlled by a separate enhancer element that is located in the first intron of the gene and does not require the *lab* promoter. Finally, the expression of *lab* in the endoderm is controlled by two separate enhancer elements and two different trans-acting members of the anterior-posterior segmentation pathway.

The anterior midgut expression requires a 500-bp fragment upstream of the start of transcription and the action of the *huckebein* locus. The posterior midgut accumulation is mediated by a 500-bp fragment in the first intron and requires the presence of the *forkhead* gene. Thus, unlike the *pb* and *Scr* genes, the regulation of *lab* appears to be entirely positive. (This work is supported by a grant from the National Institutes of Health.)

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MOLECULAR APPROACHES TO HUMAN NEUROMUSCULAR DISEASE

LOUIS M. KUNKEL, PH.D., *Investigator*

Previous reports have detailed the identification of dystrophin as the protein altered by mutation to yield Duchenne and Becker muscular dystrophy. In an effort to characterize the consequences of abnormal dystrophin in diseased tissues, Dr. Kunkel and his colleagues have refined their analysis of the distribution of dystrophin in those tissues that normally express it and have continued to characterize some of the nondeletion mutations that yield abnormal dystrophin. To further the understanding of dystrophin function and its disruption in disease, the laboratory has made a major effort to characterize additional cytoskeletal proteins that might act in tandem with dystrophin in muscle tissue. These dystrophin-related proteins might play some role in mitigating the consequences of absent dystrophin and are themselves prime candidates to be altered in other neuromuscular diseases.

The 427-kDa form of dystrophin has been shown in this and other laboratories to be expressed in smooth, cardiac, and skeletal muscle, as well as in the brain of both humans and mice. In collaboration with Dr. Simon Watkins, Dr. Kunkel's group has refined this localization with a sensitive and specific antibody directed against a large fusion peptide encompassing the last half of dystrophin. With this antibody the laboratory has shown, contrary to previous biochemical fractionations, that dystrophin in skeletal muscle is not associated with any internal cellular structures, but rather is solely localized to the plasma membrane.

An elevated concentration of dystrophin is found at the myotendinous junction and the neuromuscular junction, the labeling in the latter being more intense in the troughs of the synaptic folds. As in skeletal muscle, dystrophin is concentrated at the surface plasma membrane of cardiac muscle but is notably absent from the membrane areas that overlie the adherence junctions of the intercalated discs. Much less plasma membrane labeling is observed in smooth muscle and is concentrated in areas of the membrane underlain by membranous vesicles. A previous report described the postsynaptic membrane labeling of Purkinje cells of the cerebellum and a subset of cortical neurons of the cerebral cortex.

Dr. Kunkel's group has continued the characterization of alternative dystrophin mutations, especially those involved in splicing of dystrophin's huge primary transcript. The aberrant splicing of at least one exon has been documented, but the exact mutation remains elusive. Despite extensive sequences surrounding the exons involved, no nucleotide change has been documented as the cause of abnormal splicing.

The most exciting findings related to alternative exon usage have probably been the demonstration by this and other laboratories of two alternative transcription start points near the end of the gene that yield shortened dystrophin proteins lacking the amino-terminal domain and much of the rod domain of the protein. One of these begins transcription in the intron before exon 62 (work of Dr. David Yaffee in Israel) and encodes a 71-kDa protein. Dr. Kunkel's group has detected this protein with carboxyl-terminal antibodies and, like the Israeli group, finds it in many tissues, but not in muscle. Dr. Kunkel and his colleagues have also detected a 115-kDa protein with their carboxyl-terminal antibodies that was originally thought to be a related protein but has proved to be encoded from a transcription start point between exons 53 and 54 of the dystrophin gene. This protein is produced only by Schwann cells and has been detected in no other tissues. Investigation of the function of these two shorter dystrophin proteins and their pathogenic role, if any, continues.

One of the major efforts of the laboratory over the past year has been to identify the gene that is mutated to yield the degenerative motor neuron disease spinal muscular atrophy (SMA). An antigenically cross-reactive protein was detected in human and mouse brain with one of the laboratory's carboxyl-terminal dystrophin antibodies. The human form of this protein was cloned from an expression cDNA library, and when the human sequence was used to screen the GeneBank database, the encoded protein was found to be that of the previously cloned microtubule-associated protein 1B (MAP-1B). The human locus for this protein was found to be chromosome 5, the location of mutations causing SMA.

The laboratory identified two new dinucleotide repeat polymorphisms in the human MAP-1B locus, and these were shown to be linked very tightly to SMA mutations in SMA families. The tight linkage of MAP-1B to SMA, together with the known function of mouse MAP-1B in neuronal survival and outgrowth, made this a possible candidate for the SMA gene. The entire 10-kb human transcript was cloned as cDNA, and the complete sequence of the human gene determined. No gross structural alterations were found in the MAP-1B gene in 40 unrelated SMA

patients. As a result, Dr. Kunkel's group has eliminated 90% of the gene as harboring a point mutation that might cause the SMA phenotype. In addition, key recombinant individuals have been identified that place SMA mutations 5' to the MAP-1B dinucleotide repeat polymorphisms.

In collaboration with Dr. Conrad Gilliam (Columbia University), the laboratory has used the MAP-1B clone and a flanking genetic marker to screen YAC (yeast artificial chromosome) libraries. The distance between these markers is thought to be no more than 2,000 kb, and while the laboratory's YACs nearly cover this distance, they do not currently overlap. All of the reagents necessary to identify in these YACs possible genes that might be involved in SMA have been set up, including three new cDNA libraries from muscle, spinal cord, and brain, as well as a panel of SMA patient blots. The aim is to identify all possible coding sequences in the region and compare them with those from both SMA patients and normal individuals, in an attempt to identify the SMA gene.

The next year is expected to reveal new dystrophin-related proteins and what role, if any, they play in Duchenne muscular dystrophy and other neuromuscular disorders.

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MOLECULAR STUDY OF DOWN SYNDROME

DAVID M. KURNIT, M.D., PH.D., *Investigator*

Etiology

Analysis by Dr. Kurnit and his colleagues of objective molecular polymorphisms on chromosome 21 has already yielded two surprising results: 1) 94% of meiotic errors are maternal (previous subjective cytogenetic heteromorphism data, which must have been biased, indicated wrongly that only 75–80% of nondisjunction errors were maternal); 2) crossing over is either absent or, less likely, unusually telomeric on a plurality of nondisjoined chromosome 21's.

To learn why the risk of having a child with Down syndrome increases dramatically with advancing maternal age, polymorphisms that abut the centromere of chromosome 21 are required. For this purpose, the laboratory constructed a molecular probe that detects four independent pericentromeric polymorphisms on 21q. These polymorphisms result from variations in the length of oligo d(A,C):d(G,T) stretches. A fluorescent detection system has been developed and probes tailored so that all four polymerase chain reaction (PCR) polymorphisms among three family members (a father, mother, and child with trisomy 21) can be placed in a single lane of a polyacrylamide gel. Analyses of these polymorphisms will allow the inheritance of the centromeres of chromosome 21 to be followed in 600 Down syndrome families.

By determining whether the type of meiotic error (i.e., meiosis I vs. meiosis II, which can only be decided at the centromere) that yields nondisjunction shows either no maternal-age dependence or a distinct maternal-age dependence, one should be able to distinguish whether the increase with maternal age is due to relaxed selection (whereby older mothers lose the ability to select against abnormal conceptuses) or to older eggs (whereby older mothers release gametes that are more likely to have chromosomal errors). Thus the broader question of why older mothers bear more Down syndrome offspring should be answered. Furthermore, it will be possible to measure recombination across the centromere in the minority of families where short-arm markers (cytogenetic heteromorphisms and/or *in situ* hybridization with β satellite) are distinct and informative.

Pathogenesis: Recombination-based Assay and Genes on Chromosome 21

Dr. Kurnit's laboratory has developed a recombination-based assay for rapid isolation of genes on chromosome 21. In this assay chimeric phages or phasmids are isolated that have acquired a plasmid with *supF* via homologous recombination mediated by shared homology between the genic (cDNA) sequences in the phages and the genomic sequence in the plasmid. This enables one to determine if a genomic sequence is transcribed in a given tissue at a given time of development. The recombination-based assay can then be reversed by selecting against *supF* to yield the gene of interest free of the genomic sequence in the plasmid that was originally used to screen for the transcribed sequence.

Although these goals can be accomplished via hybridization, the benefits of the recombination-based assay stem from the ability to screen a large number of recombinants much more rapidly than by hybridization. Through use of the assay, a complex library encompassing 10^7 recombinants can be screened simply by plating on several petri dishes. This speed and economy will be required to append a genic initiative (i.e., a screen for transcribed sequences) onto the genomic initiative, making it possible to monitor efficiently whether a few-copy sequence is transcribed in a given tissue at a given time of development. If so, the gene of interest can be isolated by reversing the recombination-based assay.

Recombination (*supF* acquisition) is selected for on a *lacZam dnaBam*/P1 *ban*–balanced lethal host, DM21, that Dr. Kurnit and his colleagues constructed. They also constructed a pMAD supF plasmid based on R6K (not homologous to the ColE1 replicon, present in pBR322, for example) that allows screening of cDNA libraries via recombination that have sequences homologous to ColE1, including common cDNA libraries constructed in λgt10 and λgt11, and phasmid vectors such as λZAP. DM21 selects for phages that have incorporated a *supF* plasmid by homologous recombination (due to the presence of the *dnaBam* mutation) yielding blue plaques (due to the presence of the *lacZam* mutation). Furthermore, phasmid libraries can be screened using DM21 (pBR322), as the ColE1 replicon in DM21 (pBR322) is incompatible with and thereby inhibits replication driven by the ColE1 replicon in phasmid vectors. Excision during reversal of the selection process (counterselection) yields the genic (cDNA) insert free of the genomic insert in pMAD. Counterselection of inserts selected from phage vectors is achieved by PCR application, followed by cloning into a pBLUESCRIPT plasmid. Counterselection of inserts selected from phasmid

vectors is achieved by M13-mediated coinfection of the phasmid-carrying strain, first to isolate the pMAD/genomic-ColE1/genic plasmid cointegrate and then, by galactocide selection against *supF*, to delete the *supF*-containing pMAD/genomic sequence and obtain the desired ColE1/genic sequence. The existence of homology between a genomic and a genic sequence demonstrates that the genomic sequence encodes a transcribed region.

Dr. Kurnit and his colleagues illustrated this technology for the fragile X sequence, demonstrating that the fragile X sequence is transcribed in adult jejunum, in an 11-week fetus, and in a variety of 20-week human fetal tissues, including brain, spinal cord, eye, liver, kidney, and skeletal muscle.

Isolation of few-copy sequences. Copy number is determined by the frequency with which a given insert in a *supF* plasmid retrieves sequences from a genomic library constructed in phage λ. A higher degree of recombination is associated with a higher degree of repetition. This rapid methodology enables separation of few-copy, moderately repetitive, and highly repetitive sequences. Since hybridization with radiolabeled human DNA does not distinguish few-copy from moderately repetitive sequences, the technique is essential for rapid analysis of copy number in this range. The strategy has been used to construct a multiplex nonrepeated probe for the distal region of chromosome 21 that should be useful for prenatal diagnosis.

Determination of the transcriptional activity of few-copy fragments in different tissues at different times, coupled with concomitant isolation of the gene. A bank of human fetal (brain, spinal cord, eye, kidney, and voluntary muscle) and HeLa cDNA libraries was screened via recombination with few-copy genomic probes (see above) subcloned from several YACs localized to the distal region of 21q22, which is responsible for many of the phenotypic defects seen in Down syndrome. Whether transcription occurred in particular tissues at particular times of development was thereby determined. Seventy transcripts (some of which must overlap) have been rescued. Many of the clones were represented in multiple cDNA libraries.

The high degree of transcription observed via recombination is in accord with previous hybridization results in which 12 out of 20 few-copy sequences were represented in a complex library of

fetal brain cDNA that Dr. Kurnit's laboratory constructed. These results indicate that a significant proportion of single-copy sequences are transcribed. The ease, generality, and rapidity of application combine to make the recombination-based assay a method of choice for coupling a genic initiative to the genomic initiative. This permits efficient screening for the time and tissue of transcription as well as isolation of the transcribed sequences.

The laboratory's finding that a significant plurality of a large number of few-copy sequences are transcribed, which was not feasible by other technologies, has several corollaries:

1. Since a considerable proportion of the few-copy segment of the genome is transcribed, the search for genes must be efficient. This point reinforces the need for a rapid assay to search for transcription in multiple gene libraries.

2. As a necessary corollary to the complexity of transcription in point 1, the transcription that occurs is seldom abundant. If many different sequences are transcribed, then the frequency with which each is present in a gene library must be low.

3. It is incumbent to decipher the timing and tissue of transcription of a given sequence. The presence of clones in some but not all cDNA libraries underscores the necessity of searching for transcription in different libraries.

Dr. Kurnit is also Professor of Pediatrics and Human Genetics at the University of Michigan Medical School.

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BIOLOGY OF HUMAN PAPILLOMAVIRUSES

Laimonis A. Laimins, Ph.D., *Assistant Investigator*

Dr. Laimins and his colleagues are studying the biology of human papillomaviruses (HPVs). Areas of interest include 1) transformation of human epithelial cells, 2) regulation of viral gene expression, and 3) propagation of HPV in culture.

Papillomaviruses are small DNA viruses that infect epithelial cells and induce hyperplasias in most mammals, including humans. More than 60 different types of HPVs have been identified to date, and one-third of these are specific for genital epithelium. A subset of these viruses (notably types 16, 18, and 31) are the etiological agents of cervical and other anogenital cancers. Papillomavirus infection occurs through microwounds of the epithelium, which allow entry into basal cells, where viral genomes are established as episomes. The full life cycle of HPV requires epithelial stratification, since amplification of viral genomes and capsid synthesis occur only in the terminally differentiated cells of the upper epithelial layers. This tight coupling of the viral life cycle to differentiation and the inability to duplicate these features in tissue culture may explain why these viruses have not previously been propagated in culture. Study of HPVs is also complicated by the fact that only small amounts of virus can be obtained from patients. Most studies of HPVs have therefore involved the use of cloned viral genomes isolated from biopsies of cervical cancers.

Transformation

The mechanisms by which the oncogenic HPVs immortalize human keratinocytes and alter their differentiation capabilities are being examined *in vitro*. Using human foreskin and cervical keratinocytes, the laboratory has demonstrated that two early gene products, E6 and E7, are necessary for high-frequency transformation *in vitro*. These genes are specifically retained and expressed in high-grade neoplasias and cancers *in vivo*. Although the E7 gene product can by itself extend the life span of transfected cells, the presence of the E6 protein greatly increases the rate of immortalization. The E6 and E7 gene products also alter the differentiation properties of epithelial cells. In an *in vitro* system for epithelial differentiation, referred to as the raft system, epithelial cell lines transfected with HPV-18 E6 and E7 were observed to exhibit morphological changes similar to those seen in cervical neoplasias *in vivo*. Stratified rafts of cell lines that express the E7 gene product alone exhibit minimal changes in differentiation and resemble histological

cross sections of biopsies of normal human keratinocytes. Only with the addition of the E6 protein is epithelial differentiation dramatically altered in the raft system.

The E7 gene product transforms cells through its interaction with the cell cycle regulatory protein, retinoblastoma. Dr. Laimins and his colleagues have demonstrated that the E7 protein exists as a multimeric complex that is coordinated by the presence of zinc atoms. Similarly, the E6 protein interacts with the cellular p53 protein. The laboratory has demonstrated that the binding of E6 protein decreases the half-life of p53 by two- to threefold *in vivo*. Both cell cycle regulatory proteins, retinoblastoma and p53, have been implicated in transcriptional control, suggesting a mechanism by which their normal function is altered through the binding of E7 and E6. Studies in this laboratory have demonstrated that p53 specifically represses TATA-containing promoters but does not affect initiator-mediated transcription. The E6 protein was found to abrogate this repression.

Regulation of Viral Gene Expression

Dr. Laimins and his colleagues have identified the cis sequences and trans factors that are important for the tissue-specific expression exhibited by HPVs. An HPV region of ~1 kb in length—the upstream regulatory region (URR)—has been found to contain several enhancer elements. One of these enhancers, the C enhancer, depends solely on cellular factors for activity and is a major determinant of the host range of HPV infection. This enhancer is responsible for the initial activation of viral expression following infection and is the major regulator of HPV transcription in cervical cancers. The binding of the ubiquitous AP-1 factor together with a novel keratinocyte factor, KRF-1, are necessary for C enhancer function. Furthermore, it has been observed that octamer proteins bind to sequences that overlap the KRF-1–binding site and act as repressors of HPV expression. The interaction of these factors in a stratified epithelium activates viral expression in a differentiation-specific manner.

A second enhancer in the URR is responsive to the virally encoded transcriptional activator E2. In low-grade lesions where viral genomes are maintained as episomes, E2 is the major regulator of viral expression and is thought to act as a repressor of E6/E7 transcription. In contrast, in high-grade neoplasias and cancers, viral sequences are frequently found

integrated into the host chromosome in a manner so as to disrupt E2 expression. As a result of integration, repression of E6 and E7 is removed, and high levels of the transforming proteins are expressed. The integration of viral sequences may thus be an important step in the progression of HPV-induced disease. The amino acids responsible for DNA binding and dimerization of the E2 proteins have been identified in collaborative studies with Dr. Eliott Androphy (Tufts University). The function of E2 may not be limited to transcription, as studies have shown it to form a complex with the HPV replication protein, E1. This E1-E2 complex may play a significant role in both the initiation of replication and transcriptional regulation. The interplay of viral and cellular regulators is a determining factor in both the productive stages of HPV infection and in HPV-induced cancers.

Propagation of Human Papillomavirus in Tissue Culture

Dr. Laimins and his colleagues have recently developed a method to propagate HPVs in culture. The maintenance of viral genomes as episomes is a necessary prerequisite for virus production, yet when viral sequences are transfected into human keratinocytes they quickly integrate into the host chromosome. To overcome this restriction, the laboratory has isolated cell lines derived from low-grade cervical neoplasias that contain episomal copies of HPV. When these cell lines are allowed to stratify in raft cultures *in vitro*, amplification of viral genomes is observed in the highly differentiated suprabasal cells in a manner similar to that observed in productive infections *in vivo*. In addition, differentiation-specific induction of late transcription is observed in raft cultures. Recently Dr. Laimins has been able to induce the production in culture of one of the oncogenic viral types, HPV 31b, through the addition of phorbol esters to the media. This is the first propagation of an oncogenic type of HPV in a tissue culture system. In the future this system will allow for the study of the entire viral life cycle in a tissue culture system. A grant from the American Cancer Society provided support for the project described above.

Dr. Laimins is also Associate Professor of Molecular Genetics and Cell Biology and on the Committee on Virology at the University of Chicago.

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GENETIC STUDIES IN CARDIOVASCULAR DISEASE

JEAN-MARC LALOUEL, M.D., D.SC., *Investigator*

Common cardiovascular disorders such as coronary artery disease and hypertension exhibit a familial tendency. Such broad clinical categories represent complex etiological entities, where many genes and environmental determinants are likely to be involved. Multiplicity and heterogeneity in causation stretch the ability of genetic methods to the limit. In addition to investigations of familial hyperlipidemias—where the possible contribution of two key lipolytic enzymes, lipoprotein lipase and hepatic triglyceride lipase, is being examined—research in Dr. Lalouel's laboratory also bears on the genetics of human hypertension. This report summarizes progress in the latter area. (This work was supported in part by a grant from the National Heart, Lung, and Blood Institute, National Institutes of Health.)

Human hypertension can serve as a genetic paradigm of common disease. While linkage analysis becomes increasingly attractive for an ever-broader class of familial disorders, hypertension research requires critical decisions in experimental design, particularly with respect to definition of phenotype,

model of inheritance, optimal family structures, and candidate-gene versus general-linkage approaches.

Work in this laboratory follows two directions. On the one hand, rare Mendelian syndromes are examined in the hope that unraveling their molecular basis may shed new light on the pathophysiology of commoner forms of essential hypertension. On the other hand, an attempt is made to identify genetic determinants at play in essential hypertension by applying linkage analysis in a large series of hypertensive siblings. Richard Lifton and Xavier Jeunemaitre led each project.

Glucocorticoid-remediable aldosteronism (GRA) is a rare autosomal dominant disorder marked by severe hypertension and hyperaldosteronism with high levels of abnormal adrenal steroids. All these manifestations can be corrected by the administration of glucocorticoids.

Aldosterone is a steroid involved in the regulation of sodium and potassium ion balance. It is produced in the zona glomerulosa of the adrenal gland under the primary control of the renin-angiotensin system. By contrast, glucocorticoids exert their effects on carbohydrate metabolism, are produced by the zona fasciculata of the adrenals, and are regulated by the adenohypophyseal hormone adrenocorticotropin (ACTH).

The synthetic pathways of aldosterone and the glucocorticoids share several enzymes, including 11β-hydroxylase. Aldosterone synthesis, however, requires a unique enzymatic step catalyzed by aldosterone synthase, which is normally expressed only in the zona glomerulosa. In a subject with GRA, it was found that an unequal crossing over between 11β-hydroxylase and aldosterone synthase, in close proximity on chromosome 8, had created a new chimeric gene composed of regulatory sequences of 11β-hydroxylase and sequences responsible for the catalytic specificity of aldosterone synthase.

This observation explains the ectopic production of aldosterone in the adrenal tissue responsible for synthesis of glucocorticoids and the corresponding hormonal control observed in GRA. It provides a clear interpretation of the complex physiology of a rare form of human hypertension.

Another study has been initiated dealing directly with the common phenotype of essential hypertension. Lacking a well-defined mode of inheritance, linkage is being sought in hypertensive siblings, following a protocol that does not require any assumption regarding the mode of inheritance. Rather, linkage is inferred when the number of alleles shared by hypertensive siblings at a marker locus significantly exceeds that expected under the hypothesis of independent segregation between disease and markers.

In collaboration with Dr. Roger Williams, 244 pairs of hypertensive siblings were studied and DNA samples prepared from all study subjects. Of the numerous metabolic pathways that could be involved in the pathogenesis of hypertension, the renin-angiotensin system was singled out at the outset in light of its central roles in salt and fluid homeostasis and the maintenance of vascular tone.

Stimulation or inhibition of this system respectively raises or lowers blood pressure. Consequently, each component of the system represents a potential candidate in the etiology of hypertension. In response to salt depletion or a drop in blood pressure, renin (REN), an aspartyl protease secreted by the kidney, hydrolyzes angiotensinogen (ANG) to release the decapeptide prohormone angiotensin I. Further cleavage of angiotensin I by dipeptidyl carboxypeptidase, the angiotensin-converting enzyme (ACE), produces angiotensin II, leading to vasoconstriction and increased sodium reabsorption through stimulation of aldosterone secretion. Highly informative polymorphic markers at each of these three loci have been used to perform linkage tests in hypertensive siblings. The studies with REN and ACE have been completed, and the study with ANG is under way.

Animal models have offered suggestive evidence for a role of either REN or ACE in the pathogenesis of hypertension in selected laboratory strains of rats. The possible involvement of ACE in human hypertension was suggested in two studies recently published. Both reported significant linkage between blood pressure and an anonymous marker of the growth hormone gene in the immediate vicinity of the gene encoding ACE in a strain of spontaneously hypertensive rats, the stroke-prone SHR-SP.

The potential significance of this finding was underlined by the success of ACE inhibitors in the treatment of essential hypertension in humans. A highly informative marker was developed at the human growth hormone locus, and tight linkage between growth hormone and ACE was confirmed in humans. This marker was characterized in hypertensive siblings. There was no evidence in support of genetic linkage in these siblings, whether the entire sample or subsets based on earlier onset or greater severity were selected.

Similar results were obtained at the REN locus. These negative results indicate that molecular variants of these genes do not commonly contribute to the pathogenesis of essential hypertension in humans. They also represent the beginning of a sustained investigation of the genetics of essential hypertension.

Dr. Lalouel is also Professor of Human Genetics at the University of Utah School of Medicine.

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GENETIC CONTROL OF CELL GROWTH AND DEVELOPMENT

Philip Leder, M.D., *Senior Investigator*

Dr. Leder and his colleagues are attempting to understand mechanisms that control malignant cell growth. Specific attention this year has focused on the action of certain growth factors that can act as oncogenes and other factors (lymphokines) that can modulate tumorigenesis in the living organism. Much of this work involves the transgenic mouse as an experimental paradigm.

Interleukin-4, a Biologic Regulator and Antitumor Agent

Molecular and cellular interactions of IL-4. Dr. Leder and his colleagues have pursued two lines of investigation in order to understand the potent antitumor effect of the lymphokine IL-4. Several years ago they discovered (and described in an earlier report) this antitumor effect as an indirect or host-induced response. Having now devised a convenient solid-phase assay to assess the binding activity of IL-4 to its receptor, they were able to identify those amino acids and regions of the IL-4 molecule that are required for receptor-ligand interaction.

In a separate assay viewing the effects of IL-4 on the activation of T cells, they correlated the observed binding properties with the biologic activity of mutant ligand molecules. In so doing, they defined regions and amino acid residues of the human and mouse IL-4 molecules that are essential for species-specific binding. They also identified a specific mutation capable of binding, but not of inducing, a biologic response. These studies put the evolutionary changes in the human and mouse IL-4 molecules in a rational context and created an important IL-4 mutant that can be used as a dominant negative for perturbing IL-4 function *in vivo*.

The antitumor effect of IL-4 is eosinophil dependent. Inasmuch as the antitumor effect of IL-4 is mediated via a host response mechanism, it was of great interest to determine the cellular requirements through which this effect operates. Making

use of appropriate mouse mutants that either lack or suffer depleted populations of thymus-dependent T cells (*nu/nu*), NK cells (*bg/bg*), mast cells (*W/W^v*), B cells (*B-less*), or B and T cells (SCID), Dr. Leder and his colleagues showed that these inflammatory cell types are not required for the antitumor response.

Furthermore, by noting that regressing tumors contain relatively large numbers of eosinophils (a cell type associated with parasitic infection and allergic responses), they took advantage of a monoclonal antibody targeted to eliminate eosinophils and other granulocytes. Treatment with this neutralizing antiserum greatly reduced eosinophils at the tumor site and strongly suppressed the antitumor effect. Thus they established a role for eosinophils in the antitumor response and identified a plausible avenue for therapy in human malignancy.

Growth/Differentiation Factors as Oncogenic Agents

The oncogene int-2 *and other members of the fibroblast growth factor (FGF) gene family.* Int-2 is the product of a gene very closely related to the FGF family of genes, some of which play an important role in cell growth and differentiation. *int-2* itself is normally expressed only during embryonic development and has been shown by others to be essential for the formation of the embryonic otic cup. In adult mice it has been found, again by others, to be perturbed and expressed as a result of viral insertional mutagenesis in certain mammary carcinomas. Using transgenic mice, Dr. Leder and his colleagues showed that the dysregulated *int-2* gene induces mammary hyperplasia and, ultimately, mammary carcinoma in female transgenic mice and benign prostatic hypertrophy in male mice. In an experiment with obvious clinical implications, the prostatic effect was shown to be androgen dependent and hormonally responsive.

Further detailed studies involving the transplantation of hypertrophic mammary epithelium to the mammary glands of wild-type recipient mice and similar reciprocal experiments showed that the effect of *int-2* was only active over a very short distance, several microns at most. This experiment strongly suggests that *int-2* induces mammary hyperplasia via a cell-autonomous, autocrine effect, an interpretation consistent with *int-2*'s apparent action in the developing central nervous system.

In separate studies involving transgenic mice and in collaboration with Dr. Harold Varmus's group, it was demonstrated that *int-2* cooperates with the oncogene *Wnt-1* (a secreted glycoprotein that also induces mammary hyperplasia and carcinoma in transgenic mice) to accelerate the incidence of mammary carcinoma in mice carrying both oncogenic transgenes. Inasmuch as *int-2* is a member of the FGF family of genes that has been virtually impossible to obtain in soluble form, Dr. Leder's group has continued its studies of the molecular interactions of the closely related gene *bFGF* (basic FGF) as a surrogate for *int-2*. This work has defined the heparin requirement for the interaction between bFGF and its receptor and points to a role for heparin in regulating the short-range release of such growth factors from the extracellular matrix.

Developmental Anomalies in Transgenic Mice

The role of formins in embryonic development. Dr. Leder and his group have defined a set of proteins, the formins, on the basis of their involvement in the embryonic formation of the mouse limb and kidney. Using the integration of a transgene that created an allele of the *limb deformity* (*ld*) mutation, the Leder group defined the locus as one that gives rise to a variety of alternatively spliced mRNAs, each capable of encoding a different isoform of the formin set. Recently they have characterized a new isoform that differs from those previously characterized, in that it encodes a highly acidic aminoterminal exon.

This isoform is expressed in the mesenchyme and apical ectodermal ridge of the developing embryonic limb bud, as well as the developing central nervous system and several adult tissues. The fact that the phenotypes of the *ld* alleles are restricted to the limbs and kidneys suggests that they represent only partial loss-of-function mutations, a view consistent with the observation that all the structurally characterized alleles of *ld* involve deletions of the same small carboxyl-terminal portion of the formin isoforms.

B-less, *a novel immunodeficient transgenic mutation that abrogates B cell formation.* Several naturally occurring mutant mice have been defined in which there is interference with the development of the immune system. The best characterized and most useful of these are the nude mice (thymus-dependent T cell) and SCID mice (T and B cell–deficient). Both have been used for a variety of experimental purposes, many involving transplantation studies.

In the course of experiments designed to understand expression of the human λ immunoglobulin light-chain gene, Dr. Leder and his colleagues created a transgenic mouse bearing this human gene. The mouse is dramatically deficient in B cells and, as a consequence, profoundly immunodeficient. The molecular basis for this effect, which is sup-

pressed in certain genetic backgrounds, is under investigation.

Dr. Leder is also John Emory Andrus Professor of Genetics at Harvard Medical School.

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MODELS AND APPROACHES FOR SOMATIC GENE THERAPY

FRED D. LEDLEY, M.D., *Assistant Investigator*

During the past year Dr. Ledley's laboratory has advanced the characterization of methylmalonyl-CoA (coenzyme A) mutase deficiency and has moved to animal models in the development of methods for gene therapy of this deficiency state. The laboratory has also embarked in two new directions aimed at establishing novel applications of gene therapy for congenital hypothyroidism and arthritis.

Mutations, Structure, and Function in Methylmalonyl CoA Mutase

In continuing studies of methylmalonyl-CoA mutase (MCM), a series of mutations have been identified in cell lines that exhibit an intermediate form of the deficiency state, one partially remediated by administration of pharmacological levels of the hydroxylcobalamin cofactor. These cell lines are also unique in that they participate in interallelic com-

plementation with the mutant allele R93H. Four mutations were identified that clustered at the 3′ end of the gene, close to another mutation with this characteristic phenotype that was identified previously.

Alleles with each of these mutations were shown to express an intermediate, cobalamin-responsive phenotype after gene transfer into cells that do not express MCM. Each complemented with clones having R93H in cotransformation experiments, and each expressed a mutant phenotype when overexpressed in *Saccharomyces cereviseae*. Several lines of evidence from the laboratory's studies and from studies on *Propionibacterium shermanii* MCM point to these mutations occurring within the cobalamin-binding domain of the protein. Studies are under way to confirm the assignment of this domain by direct binding studies and to identify the nature of the defect expressed by R93H.

Toward Gene Therapy for MCM Deficiency

Genetic MCM deficiency represents an attractive candidate for hepatic gene therapy. Two models have been developed.

One model involves the transduction of primary cells with retroviruses capable of permanently transforming cells with a normal MCM-containing provirus. High-titer retroviral vectors have been established that are capable of complementing the biochemical defect in mutant fibroblasts and expressing the recombinant gene product in primary human hepatocytes. Other studies in the laboratory have demonstrated that human hepatocytes can be effectively harvested, cultivated, and transplanted into immunodeficient animals, where they will engraft within the liver. The transduction of human hepatocytes with amphotropic retrovirus was found to be significantly worse than in many common animal models. Greater efficiency of transduction is obtained with xenotropic vectors that recognize a distinct receptor on human cells. Coupled with an ongoing clinical trial of hepatocellular transplantation with retroviral-marked cells, these experiments may pave the way for considering an *ex vivo* scheme for somatic gene therapy.

An alternative scheme for gene therapy involves the targeting of DNA vectors to the liver with protein-DNA complexes. In collaboration with Dr. George Wu (University of Connecticut), an MCM expression vector was coupled with asialorosomucoid via polylysine and injected into the tail vein of mice. Studies demonstrated that 1) DNA was cleared from the blood in 10–20 minutes, 2) >90% of DNA was taken up by the liver, 3) DNA was rapidly degraded by the liver and largely eliminated by 24

hours, 4) MCM mRNA was expressed in the liver 8–24 hours after administration, and 5) MCM enzyme activity was increased 25–50% over baseline (normal) between 24 and 48 hours after administration. This approach to therapy may be highly efficacious in MCM deficiency, which is characterized by an acute episode of dyshomeostasis punctuating relative stability on dietary and vitamin therapy.

Targeting to the Thyroid, a Potential for Somatic Gene Therapy

This laboratory has begun to explore methods for gene delivery to the thyroid, which represents a novel target for somatic gene therapy. The thyroid would be a particularly attractive target because it can be surgically manipulated using minimally invasive procedures, because thyroid follicular cells have an extremely large capacity for protein synthesis and are susceptible to extensive temporal regulation, and because the thyroid has a high rate of constitutive blood flow. The thyroid may be used as a target for treating monogenic forms of congenital hypothyroidism (cretinism), acquired thyroid disease, and disorders requiring constitution of hormones or serum proteins in the blood.

Two methods for gene delivery to the thyroid have been established. The first involves a classic *ex vivo* scheme. In dogs, thyroid follicular cells have been harvested, cultivated in a serum-free media, transduced with retroviral vectors, and transplanted into autologous animals. Studies have shown that follicular cells preserve differentiated thyroid functions, can be selected for G418 resistance after transduction with NEO-R–containing retroviruses, and express the recombinant gene product *in vivo*.

The thyroid can also be transiently transformed by direct injection of DNA vectors into the body of the gland. In rabbits, injection of vectors expressing chloramphenicol acetyltransferase leads to high levels of gene expression for 3–5 days. Studies with β-galactosidase indicate that thyroid follicular cells are expressing the recombinant gene. The thyroid thus shares with muscle the unusual property of effectively taking up and expressing recombinant genes after injection of naked DNA. Thus gene therapy directed at these organs may be considered, using genes as medicines that will resemble conventional pharmaceuticals in their mode of delivery, metabolism, and pharmacology.

Direct Gene Delivery to Joints: Potential Gene Therapy for Arthritis

Studies in Dr. Ledley's laboratory have also been directed at assessing methods for transferring genes into the synovium of joints as a means of treating

arthritis. Direct injection of vectors expressing β-galactosidase has been shown to lead to selective expression of the recombinant gene product in synovial cells. The hypothesis is that in joints, as in the thyroid, injection of DNA into a fluid compartment that is normally remodeled by pinocytosis will lead to gene expression in cells reabsorbing the fluid. Studies are under way to extend these observations to generate animal models of arthritis as well as methods for treating various forms of arthritis with gene medicines.

The project described above was supported by a grant from the National Institutes of Health.

Dr. Ledley is also Associate Professor of Cell Biology and Pediatrics at Baylor College of Medicine and a member of the Section on Gastroenterology and Nutrition at Texas Children's Hospital.

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EUKARYOTIC GENE EXPRESSION

Stephen A. Liebhaber, M.D., *Investigator*

Dr. Liebhaber's laboratory is studying the expression and function of the human α-globin gene and growth hormone gene clusters. These studies address both transcriptional and post-transcriptional controls critical to the high-level, tissue-specific, and developmentally controlled expression of these genes. An additional and overlapping area of investigation that utilizes these two gene clusters as model systems is the study of the relationship of RNA structure to its processing and function.

Structural Determinants of Human α-Globin Gene Expression

Globin genes are expressed exclusively in differentiating erythroblasts, where levels of globin mRNA constitute >90% of total mRNA. In addition to this remarkably specific and high-level expression, the expression of individual genes within the cluster is developmentally controlled so as to maximize the fit between their oxygen-binding activities and changing ambient oxygen tensions. Studies in this laboratory focus specifically on the expression of the human α-globin gene cluster that contains an embryonic (ζ)-globin gene and two coexpressed fetal/adult α-globin genes.

Recent studies of RNA from embryonic erythroid tissue have demonstrated that the switch from ζ- to α-globin gene expression occurs between the sixth and eighth week of gestation. To study this switch in further detail, Dr. Liebhaber and his colleagues have reconstituted the entire human α-globin gene cluster along with an adjacent chromatin activation do-

main (locus control region) in transgenic mice. The human α-globin transgenes in these mice are appropriately expressed, the ζ-globin gene during the embryonic period and the α-globin gene in the fetal/adult stage. Analysis of mouse lines carrying more-limited segments of the cluster demonstrate that this developmental control is autonomous; i.e., the normal expression pattern of the α-globin gene is not dependent on the presence of the embryonic (ζ)-globin gene in cis.

These studies have been extended by generating transgenic lines carrying more-limited segments of the α- and ζ-globin genes or chimeric genes in which the α- and ζ-globin gene promoters are either reciprocally exchanged or placed under the transcriptional control of a nonerythroid reporter gene. These studies are aimed at defining the limits of the cis-acting developmental control elements.

Additional insight into the developmental switching in the α-globin gene cluster has been derived from concurrent analysis of naturally occurring α-thalassemic mutations. In these studies it was shown that a subset of large deletions within the α-globin cluster can result in persistence of ζ-globin gene expression. These data suggest that some of the determinants of ζ-globin gene silencing may be located as much as 10 kb 3' of the ζ-globin gene itself.

The selective enrichment of globin mRNAs in the developing erythroblast is dependent on the unusual stability of globin mRNAs. Based on the analysis of informative α-thalassemic mutations that destabilize globin mRNA, it was hypothesized that critical determinants of α-globin mRNA stability are located in the 3'-nontranslated region. This hypothesis was confirmed by studies using a gene transfection system in which normal or mutated human α-globin genes could be expressed and the stability of their encoded mRNAs determined. Stability determinants mapped by two approaches, a ribosome interference assay and linker scanning mutagenesis, gave mutually confirming results that revealed the existence of at least two closely spaced stability determinants. Their mode of action is now being studied.

Structure and Function of the Human Growth Hormone Genes

As with the globin gene system, the human growth hormone (hGH) gene cluster is highly regulated. The cluster contains the single gene hGH-N, expressed in the pituitary, and four genes expressed in the placenta, hGH-V, hCS-A, hCS-B, and hCS-L. Two of these five genes, hGH-V and hCS-L, initially thought to be pseudogenes, have now been shown to be expressed placentally, based on work from this laboratory. By a series of receptor-binding and bioactivity assays, hGH-V was demonstrated to represent the major somatogen hormone of pregnancy. hCS-L was demonstrated to be expressed in the placenta in a number of alternatively spliced forms, at least one of which is likely to be expressed as a secreted hormone.

Since the placenta expresses a spectrum of hGHs, it is possible that it also contains a corresponding set of hGH receptor isoforms. Consistent with this hypothesis, work from Dr. Liebhaber's laboratory has revealed the existence of an hGH receptor isoform generated by alternative splicing of exon 3 of the hGHR transcript. The deletion of exon 3 from the placentally expressed hGHR (hGHRd3) may be quite significant in that the deleted segment is located in the extracellular domain and may affect ligand binding. This alternative splice is tissue specific. Studies now in progress are aimed at defining whether hGHRd3 demonstrates a binding preference for any of the hGH-related hormones and whether it might in this way serve as the effector arm of a placental autocrine/paracrine loop. (This work was carried out in collaboration with Dr. Nancy Cooke, with funding from the National Institutes of Health.)

RNA Structure and Function

An underlying interest of this laboratory that overlaps with the studies of the globin and hGH gene systems is the study of RNA structure-function relationships. A recently completed analysis of alternative splicing of the hGH gene transcript demonstrated a direct controlling effect of transcript secondary structure. A specific stem that encompasses the major splice acceptor of the hGH-N transcript and its probable lariat branch point favors the utilization of a more distal acceptor.

Secondary structures may also affect mRNA translation. This has been documented by demonstrating that the effects of intramolecular mRNA duplexes on translation are position dependent. Intramolecular duplexes within the 5'-nontranslated region or in the 5' proximal coding region block assembly of the complete 80S ribosome, while duplexes located within the coding region well 3' to the AUG are efficiently melted out by the elongating ribosome. These data suggest that antisense strategies aimed at translational inhibition must be appropriately targeted.

In addition to their effects on mRNA processing and function, secondary structures can result in the alteration of primary structure. It was demonstrated that intramolecular duplexes in an mRNA can target a cellular adenosine → inosine converting activity. This editing activity can be targeted to a specific

segment of RNA if that region is involved in an mRNA duplex as short as 15–20 base pairs. Maximal activity (50% A → I conversion) occurs with a duplex target of 100 base pairs or greater. Although the natural function of this RNA-modifying activity remains to be delineated, these studies demonstrate that mRNA secondary structures can target this ubiquitous RNA-editing activity.

Dr. Liebhaber is also Professor of Genetics and Medicine at the University of Pennsylvania School of Medicine.

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FUNCTION AND REGULATION OF THE HEAT-SHOCK RESPONSE

Susan L. Lindquist, Ph.D., *Investigator*

When cells of all types are exposed to mildly elevated temperatures, ethanol, anoxia, heavy metal ions, or a wide variety of other stresses, they respond by producing a small number of proteins called the heat-shock proteins (HSPs). This response is one of the most highly conserved genetic regulatory systems known. Dr. Lindquist's research program focuses on 1) investigation of the functions of the HSPs in protecting cells from the toxic effects of stress and in normal growth and development and 2) use of the response as a model system to investigate mechanisms of genetic regulation, particularly post-transcriptional regulation.

HSP Function

Studies of the molecular functions of HSPs have concentrated on the yeast *Saccharomyces cerevisiae*, because of the genetic methods of analysis possible in this organism. This year Dr. Lindquist and her colleagues have concentrated on the analysis of hsp90 and hsp100, the two largest HSPs in yeast.

Yeast cells encode two closely related proteins in the hsp90 family, hsp82 and hsc82. Disruption of the gene encoding either protein prevents growth at high temperatures, while deletion of both is lethal. Thus hsp90 is a vital protein that is required at higher concentrations for growth at higher temperatures. The mammalian hsp90 gene can compensate for the loss of both yeast genes, demonstrating that the functions of these proteins have been highly conserved. This is important, because the mammalian protein has been characterized extensively at the biochemical level, and Dr. Lindquist's laboratory wishes to take advantage of yeast genetic methods to investigate such functions *in vivo*. In collaboration with Dr. Keith Yamamoto, Dr. Lindquist's laboratory demonstrated that hsp90 is required for the formation of functional steroid hormone receptors. This proved that the interaction between hsp82 and steroid receptors is important *in vivo*. It also caused a substantial revision in thinking about hsp90 functions, since hsp90 had previously been characterized as a repressor of receptor function.

This year Drs. Yamamoto and Lindquist explored the interaction between hsp90 and p60$^{v\text{-}src}$. As with the steroid receptors, the results indicate that hsp90 is essential for the formation of active kinase. Moreover, the expression of src kinase in yeast cells causes a cell cycle arrest. This indicates that src has specific targets in yeast cells. Because of the conservation of cell cycle regulation, the identification of this target may be relevant to vertebrate oncology. This work is being supported by grants from the National Institutes of Health and the Human Frontiers in Science Program.

This past year Dr. Lindquist's laboratory has increasingly focused attention on hsp100. Cells carrying mutations in the gene encoding this protein grow normally at both high and low temperatures, but are 100- to 1,000-fold more sensitive than wild-type cells to heat killing at 50°C and to ethanol killing at concentrations of 20%. The protein is very highly conserved, and heat-inducible members of the family are found in bacteria and mammals. Surprisingly, they are not found in *Drosophila*. This year the laboratory discovered that *Drosophila* cells make a heat-inducible transcript that is homologous to hsp100, indicating that expression of the protein was lost recently in the evolution of these flies. Dr. Lindquist and her colleagues are currently trying to determine how widespread this loss is among insects and if the loss of the protein might confer a growth advantage that would compensate for the presumed disadvantage in thermotolerance. The hsp100 proteins have two nucleotide-binding sites, and both are required for thermotolerance. The purified protein is an ATPase and assembles into a hexamer in the presence of ATP. Currently the laboratory is attempting to determine what substrates hsp100 interacts with and what effects mutations in the nucleotide-binding sites have on substrate interactions and self-assembly. To define proteins that might interact with hsp104, the laboratory has initiated a genetic search for dominant negative mutations in hsp104. Several have been produced and are now being characterized. Finally, mutations are being produced in another member of the hsp100 family in yeast, in collaboration with Dr. Chris Davies (University of North Carolina).

This year Dr. Lindquist and her colleagues have expanded a new line of research aimed at determining the function of HSPs in protecting animals from the developmental anomalies that are caused by high temperatures. They have developed a new system for site-directed recombination in *Drosophila* and are using it to create isogenic flies carrying various copy numbers of HSP genes and gene mutations. Flies carrying 10 extra copies of the hsp70 gene survive heat shocks better than wild-type flies, at least in the early embryonic stages, indicating that

it should be possible to manipulate the thermotolerance of complex multicellular organisms by deliberate genetic intervention.

Regulation of HSP Expression

The heat-shock response of *Drosophila* cells is particularly intense. Within minutes of a shift from 25°C to 37°C, the entire pattern of protein synthesis is shifted from the production of normal cellular proteins to the production of HSPs. After heat shock, the full pattern of normal protein synthesis is restored. In the past year the laboratory's studies in *Drosophila* have concentrated on the regulation of hsp70 synthesis. This protein is almost undetectable in cells growing at normal temperatures but, after heat shock, it is the most abundantly synthesized protein. The hsp70 message is very stable during heat shock, but during recovery it is rapidly degraded. This degradation appears to be highly specific and occurs while most other cellular messages are being reactivated for translation. Degradation is also highly regulated and only occurs after a specific quantity of protein—a quantity appropriate to the particular level of heat stress applied to the cells—is produced. When the hsp70 message was expressed at normal temperatures, from a heterologous promoter, it was found to be very unstable. Thus heat shock inactivates a preexisting mechanism for degradation, and recovery restores it.

The existence of so many regulatory mechanisms to ensure that hsp70 is expressed as rapidly as possible after heat shock but is not expressed at normal temperatures suggested that hsp70 might be toxic at normal temperatures. Using heterologous promoters, the laboratory found that hsp70 blocks the growth of *Drosophila* cells. Remarkably, after several days of ectopic expression, cells resume growth and hsp70 is found sequestered in granule-like structures in the cell. These granules apparently represent a new mechanism for regulating hsp70, in this case by controlling its activity rather than its level of expression. In the natural life cycle, this mechanism is used to regulate hsp70 activity. Within the first 3 h of embryonic life, hsp70 cannot be induced in embryos, and embryos are extremely sensitive to heat. From 3 to 6 h, hsp70 is inducible and embryos are capable of acquiring tolerance to heat. However, when embryos are allowed to recover at 25°C, they lose tolerance within a few minutes. On a similar time scale, hsp70 coalesces into granules. The laboratory speculates that although hsp70 is beneficial for thermotolerance, it is toxic to the rapid cell divisions that occur in the early embryo. For this reason expression is prohibited in the earliest embryos, and the protein is rapidly sequestered in later embryos. Future experiments will test this hypothesis and explore the mechanisms that are employed in hsp70 sequestration.

Dr. Lindquist is also Professor of Molecular Genetics and Cell Biology at the University of Chicago.

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CONTROL OF CELL SURFACE OLIGOSACCHARIDE EXPRESSION

JOHN B. LOWE, M.D., *Assistant Investigator*

During the past year, Dr. Lowe and his colleagues have continued to focus on the use of gene transfer systems to explore the functions of cell surface oligosaccharide determinants and on the isolation of additional mammalian glycosyltransferase genes. Work is also in progress to define the structures and expression patterns of murine glycosyltransferase genes, to provide a background for transgenic animal studies designed to uncover functions of cell surface oligosaccharides during development.

Mammalian cells express a diverse array of oligosaccharide molecules on their surfaces. These mole-

cules consist of carbohydrate structures covalently linked to membrane-associated proteins and lipids. Many of these structures undergo striking changes during development and differentiation and in association with neoplastic transformation. Numerous experimental observations suggest that cell surface oligosaccharides function as information-bearing molecules that mediate communication between cells and their environment during development and differentiation. The structure of cell surface oligosaccharides is determined primarily by glycosyltransferase enzymes. Molecular mechanisms responsible for the regulation of expression of these enzymes, and thus the expression of cell surface glycoconjugate structure, remain poorly defined. Moreover, in most instances the precise function(s) of the oligosaccharide structures determined by these enzymes is also obscure. The major goals of Dr. Lowe's research efforts are to understand 1) the mechanisms that regulate expression of cell surface oligosaccharide antigens and 2) the functions of these molecules, through approaches involving the use of cloned glycosyltransferase gene segments.

Dr. Lowe's laboratory is investigating representative human and murine glycosyltransferase genes as models to understand glycosyltransferase structure and regulation and the function(s) of their oligosaccharide products. These enzymes include fucosyltransferases, whose expression is determined by the human H and Lewis blood group genes, and structurally similar genes. The genetics of these systems are well understood yet informative; interesting alleles exist at these loci that will serve to elucidate relationships between the substrate specificities and primary structures of glycosyltransferases. Moreover, these enzymes and their structures are expressed in a tissue-specific and developmentally regulated manner, and their expression is often altered in association with neoplastic transformation.

To circumvent difficulties associated with conventional cloning approaches, Dr. Lowe and his colleagues have developed gene transfer strategies to isolate glycosyltransferase genes. These strategies use existing information about the substrate and acceptor properties of these enzymes and take advantage of antibody and lectin reagents specific for the surface-expressed oligosaccharide products of these enzymes. Several mammalian glycosyltransferase genes have been isolated, including the human H blood group locus, a family of $\alpha(1,3)$fucosyltransferase genes that includes the Lewis blood group locus, and a murine gene with structural and functional similarity to the human ABO blood group locus. Dr. Lowe's laboratory is continuing to use gene transfer approaches to isolate new human and mouse glycosyltransferase genes. Ongoing efforts are also focused on characterizing murine homologues of the human genes, in preparation for transgenic animal experiments designed to determine the function of their cognate oligosaccharide determinants during embryogenesis.

Dr. Lowe and his colleagues have also been investigating the roles of cell surface oligosaccharides during the inflammatory process. During inflammation in humans, neutrophils and monocytes must leave their normal locations in the intravascular space to arrive at extravascular destinations. The initial step involves adhesion between these circulating myeloid cells and the protein E-selectin (previously known as endothelial leukocyte adhesion molecule 1 [ELAM-1]), which is expressed by vascular endothelial cells. E-selectin is normally absent from quiescent endothelial cell surfaces but is expressed prominently in association with inflammatory stimuli. Like other members of the selectin family of cell adhesion receptors, E-selectin maintains an amino-terminal protein domain similar to a consensus sequence previously defined for C-type carbohydrate-binding proteins, or lectins. Although it had been suspected that E-selectin mediates adhesion of neutrophils and monocytes by interacting with an oligosaccharide ligand on the surfaces of those cells, confirmation of this and the precise nature of such a ligand had been undefined. By transfecting various cultured cell lines with specific fucosyltransferase genes and other glycosyltransferase gene segments and cDNAs, Dr. Lowe and his colleagues were able to demonstrate that members of the family of $\alpha(2,3)$sialylated, $\alpha(1,3)$fucosylated lactosaminoglycans, represented by the sialyl Lewis x oligosaccharide determinant, function as ligands for E-selectin.

These observations predicted that these oligosaccharides, or their molecular mimics, might prove useful as anti-inflammatory pharmaceuticals, by blocking adhesion of myeloid cells to E-selectin expressed by inflamed endothelium. Two approaches that avoid difficulties inherent in chemical synthesis of oligosaccharides were implemented to create oligosaccharides for testing this hypothesis. One method uses a recombinant $\alpha(1,3)$fucosyltransferase for enzyme-assisted *in vitro* synthesis of the sialyl Lewis tetrasaccharide. The other method involves the isolation of sialyl Lewis x–positive oligosaccharides from a cultured mammalian cell line transfected with an $\alpha(1,3)$fucosyltransferase gene. Representatives of each of these two types of sialyl Lewis x oligosaccharide have been tested recently, in experiments done in collaboration with Dr. Peter Ward and his colleagues (University of

Michigan). This work has demonstrated that sialyl Lewis x–containing oligosaccharides function as relatively potent anti-inflammatory molecules in animal models of selectin-dependent inflammation. Future work will focus on defining the structural requirements for anti-inflammatory activity and on exploring the ability of these molecules to abrogate selectin-dependent cell adhesion processes necessary for several types of acute and chronic inflammation.

During the past year, experiments in Dr. Lowe's laboratory have defined the molecular basis for null alleles at the human H blood group locus, which corresponds to an $\alpha(1,2)$fucosyltransferase gene previously isolated by his group. These studies confirm the hypothesis that the human genome maintains other $\alpha(1,2)$fucosyltransferase genes, one of which probably corresponds to the human Secretor blood group locus. Efforts are under way to isolate such genes, to study the biosynthesis of their enzyme products, and to define their expression patterns. Work on the human $\alpha(1,2)$fucosyltransferase genes will be supported by a grant from the National Institutes of Health.

Also during this past year, Dr. Lowe's laboratory has isolated two new human $\alpha(1,3)$fucosyltransferase genes. These represent the second and third members of a family of structurally and functionally related $\alpha(1,3)$fucosyltransferase genes. The enzymes encoded by the most similar pair of these genes are nearly identical in sequence throughout substantial portions of their lengths. These enzymes nonetheless maintain a discrete and structurally diverse polypeptide segment interspersed within the conserved sequence. These observations, together with the similarities and differences in the catalytic properties of these enzymes, suggest that the structurally diverse peptide segments play pivotal roles in determining the efficiencies with which any one of several potential oligosaccharide substrates will be utilized by each distinct fucosyltransferase. Experiments are in progress to test this hypothesis, as are studies designed to define the normal expression patterns of these human genes and to delineate the

biosynthesis of their polypeptide products. The latter studies will be supported by a grant from the National Institutes of Health.

Dr. Lowe is also Associate Professor of Pathology at the University of Michigan Medical School.

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HUMAN RETROVIRUSES AND CELLULAR GENE EXPRESSION

GARY J. NABEL, M.D., PH.D., *Associate Investigator*

The replication of viruses in a susceptible host is critically dependent on host cell factors essential to the function of eukaryotic cells. These factors often function to regulate cell proliferation and differentiation. This laboratory previously showed that a cellular transcription factor is responsible for transcriptional activation of the human immunodeficiency virus (HIV) in T lymphocytes and macrophages.

Activation of T cells, which increases HIV expression up to 50-fold, correlates with the induction of a DNA-binding protein, NF-κB. Many cellular genes that are regulated by NF-κB have been identified, and the mechanism of transcriptional activation by NF-κB defined. Furthermore, the role of NF-κB in HIV activation in T cells and monocytes has now been established, and the identification of genes encoding κB-binding proteins achieved. Knowledge derived from the study of retroviral and cellular gene expression has also been adapted to deliver recombinant genes to specific sites *in vivo*. These studies therefore address mechanisms of viral gene regulation, provide insight into the regulation of eukaryotic gene expression *in vivo*, and contribute to the treatment of human disease.

T Cell Activation and HIV Replication

The transcription factor NF-κB is a protein complex comprising a DNA-binding subunit of $\sim$50 kDa and an associated transactivation protein of $\sim$65 kDa. Both the 50- and 65-kDa subunits have similarity with the *rel* oncogene and the *Drosophila* maternal-effect gene *dorsal*. The 50-kDa DNA-binding subunit was previously thought to be a unique protein derived from the 105-kDa gene product, p105. This laboratory has isolated a complementary DNA that encodes an alternative DNA-binding subunit of NF-κB.

The new subunit is more similar to p105 NF-κB than are other family members and defines a new subset of *rel*-related genes. Synthesized as an $\sim$100-kDa protein, p100 is expressed in different cell types, contains cell cycle or ankyrin motifs, and (like p105) must be processed to generate a 50-kDa form.

A 49-kDa product (p49) can be generated independently from an alternatively spliced transcript. It has specific κB DNA-binding activity and can form heterodimers with other *rel* proteins. In contrast to the $\sim$50-kDa protein derived from p105, p49 acts in synergy with p65 to stimulate the HIV enhancer

in transiently transfected Jurkat cells. Thus p49/p100 NF-κB could be important in the regulation of HIV and other κB-containing genes.

Because it is possible that specific interactions of different subunits can allow selective gene activation, the specificity of transcriptional activation by different combinations of these subunits has now been characterized. The laboratory finds that an $\sim$50-kDa form of p100 (p49) binds weakly to κB alone but associates with p65 to bind efficiently to this site. Furthermore, p49 acts in combination with either p65 or a Rel-VP16 fusion protein to activate κB-dependent transcription in Jurkat T leukemia cells.

The p49-p65 or p49-Rel combination stimulated transcription mediated by the canonical κB site but did not stimulate interleukin-2 receptor-α (IL-2Rα) or histocompatibility complex κB reporters, despite its ability to bind to these sites. Transactivation mediated by the p49/p100 and p65 NF-κB products is therefore sensitive to minor changes in the sequence of the κB site. Specificity determined by the association of NF-κB subunits provides a mechanism for selective regulation of variant κB sites associated with different cellular and viral genes.

NF-κB also functions in concert with the *tat*-I gene product to stimulate HIV transcription. To determine whether specific members of the NF-κB family contribute to this effect, Dr. Nabel and his colleagues examined the abilities of different NF-κB subunits to act with Tat-I to stimulate transcription of HIV in Jurkat T leukemia cells. They found that the p49(100) DNA-binding subunit, together with p65, can act in concert with Tat-I to stimulate the expression of HIV-CAT (chloramphenicol acetyltransferase) plasmid. Little effect was observed with 50-kDa forms of p105 NF-κB or rel, in combination with p65 or full-length c-rel, which do not stimulate the HIV enhancer in these cells. These findings suggest that the combination of p49(100) and p65 NF-κB can act in concert with the *tat*-I gene product to stimulate the synthesis of HIV RNA.

The chromosomal locations of the human genes NFKB1 and NFKB2, which encode two alternative DNA-binding subunits of the NF-κB complex, p105 and p49/p100, respectively, have been determined. Through Southern blot analysis of panels of human–Chinese hamster cell hybrids, p105 was assigned to 4q21.1-q24 and p49/p100 to chromosome 10. The locations were confirmed by fluorescence *in situ* hybridization and mapped with

greater resolution to 4q23 and 10q24, respectively. These results demonstrate that these members of the NF-κB family are unlinked. Interestingly, p49/p100, as well as p105, maps to regions associated with certain types of acute lymphoblastic leukemia.

Transcriptional activation of the IL-2Rα gene in T cells is dependent on a regulatory element that can bind NF-κB but which differs in sequence and function from the κB site of the immunoglobulin (Ig) enhancer. To define the molecular basis of gene-specific regulation by this variant κB site, the laboratory has used electrophoretic mobility shift assays to characterize a novel gene product, designated RκB, that binds preferentially to the related κB site in IL-2Rα. A cDNA encoding this 107-kDa protein has been isolated from a λgt11 expression library by screening with a probe containing the IL-2Rα κB site.

RκB is a tissue-specific transcription factor containing an amino acid sequence similar to a discrete region of the myogenic regulatory protein MyoD, but is unrelated to other DNA-binding proteins, including those belonging to the *rel/dorsal* gene family. This novel κB-binding protein may therefore contribute to the regulation of distinct cellular and viral genes with variant κB sites. Grants from the National Institutes of Health provided partial support for the projects described above.

Gene Transfer *in Vivo*

In addition to studies of gene regulation, gene transfer techniques have been used to deliver recombinant genes to specific sites *in vivo*. Previously, endothelial or vascular smooth muscle cells were transduced with recombinant genes *in vitro* and introduced stably on porcine arteries *in vivo* after delivery with a catheter. More recently, a recombinant β-galactosidase gene was expressed in a specific arterial segment *in vivo* by direct infection with a murine amphotropic retroviral vector or by DNA transfection with the use of liposomes. Several cell types in the vessel wall were transduced, including endothelial and vascular smooth muscle cells for at least five months, and no helper virus was detected.

Autoimmune vasculitis represents a disease characterized by focal inflammation within arteries at multiple sites in the vasculature. Therapeutic interventions in this disease are empirical and often unsuccessful, and the mechanisms of immune injury are not well defined. The direct transfer of recombinant genes and their expression in the arterial wall provides an opportunity to explore the pathogenesis and treatment of vascular disease.

An animal model for vasculitis has been developed, using these direct gene transfer approaches. Inflammation has been elicited by introduction of a foreign class I major histocompatibility complex (MHC) gene, *HLA-B7*, to specific sites in porcine arteries. Transfer and expression of this recombinant gene was confirmed by polymerase chain reaction (PCR) analysis and immunohistochemistry, and cytolytic T cells specific for *HLA-B7* were detected. These findings demonstrated that expression of a recombinant gene in the vessel wall can induce a focal immune response and suggest that vessel damage induced by cell-mediated immune injury can initiate vasculitis.

The safety and toxicity of this method of gene delivery have been assessed after *in vivo* administration, either by intravenous or direct intratumor injection. Nine to eleven days after intravenous injection, DNA was found primarily in heart and lung tissue by PCR analysis. No abnormalities were evident from histologic examination of tissue, examination of tissue-specific serum enzymes, routine biochemical parameters, or electrocardiographic monitoring. DNA-liposome complexes can therefore be used for the delivery of recombinant genes *in vivo* with minimal toxicity. (These studies have been supported in part by grants from the National Institutes of Health.)

Taken together, these results have demonstrated that site-specific gene expression can be achieved by cell-mediated and direct gene transfer *in vivo* and could be applied to the treatment of such human diseases as atherosclerosis or cancer.

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MOLECULAR BIOLOGY OF THE CONTRACTILE SYSTEM

Bernardo Nadal-Ginard, M.D., Ph.D., *Investigator*

During the past year Dr. Nadal-Ginard's research has focused on three main aspects of the contractile system: 1) developmental biology of the skeletal and cardiac muscle cell, 2) the biochemistry of the terminally differentiated state, and 3) the regulatory aspects of alternative splicing by which a single gene can generate multiple protein isoforms.

Developmental Biology of Cardiac and Skeletal Muscle Cells

Although significant progress has been made in understanding the mechanisms leading to the conversion of a mesenchymal precursor to a terminally differentiated skeletal muscle cell, many of the molecular events in this cascade remain to be elucidated. Moreover, despite the phenotypic similarities among skeletal, cardiac, and, to a lesser extent, smooth muscle, the significance of the mechanisms operative in skeletal muscle for the production of cardiac and smooth muscle phenotypes is questionable. For this reason there has been a search, unsuccessful until now, for factors that would regulate tissue-specific gene expression in all three muscle cell types. A candidate for such a factor is the myocyte-specific enhancer-binding factor 2 (MEF2), an activity in muscle cell extracts that binds to an A/T reach sequence present in most, if not all, muscle-specific enhancers. Deletion of this sequence drastically reduces the transcription activity of the corresponding gene.

During the past year the laboratory has used a consensus MEF2 DNA-binding site to screen expression libraries and isolate different cDNAs encoding the MEF2 factor from skeletal and cardiac tissues. The corresponding mRNAs are the products of four different but closely related genes that, through alternative splicing, encode at least 12 different proteins. These proteins share domains strikingly homologous to the DNA-binding and dimerization domains of a recently identified MADS gene family.

The pattern of expression of these four genes is different. Two are expressed ubiquitously at the mRNA level. The protein, however, is present exclusively in skeletal and cardiac muscle cells as well as neurons. This discrepancy between mRNA and protein expression is directly correlated to the pattern of alternative splicing of these two genes. The presence of certain exons is restricted to those cells where the protein is detectable. Therefore, posttranscriptional processes and, in particular, alternative splicing are essential for the proper expression of these two genes.

In contrast, the other two genes have a very restricted pattern of expression. One is expressed, at the mRNA and protein level, preferentially in cardiac cells and neurons. The other is expressed in skeletal muscle and cortical neurons. With the assay systems available, no functional differences have been detected among the different isoforms. Cardiac and smooth, as well as skeletal, muscles contain functionally saturating levels of MEF2-transactivating factors that are absent from the non-muscle cells. Moreover, MEF2 is induced in non-muscle cells by MyoD; however, MEF2 alone is insufficient to produce the full muscle phenotype. These results indicate that, although MEF2 is induced in skeletal muscle by the MyoD family of regulators, other lineage-determining pathways must lead to MEF2 expression in nonskeletal muscle tissue, where the MyoD family is not expressed. Whether basic helix-loop-helix (bHLH) or other factors induce the MEF2 genes in cardiac and smooth muscle, as well as neurons, the regulatory sequences of these genes will serve as powerful tools for the dissection of the lineage-dependent pathways in these cell types.

Biochemistry of the Terminally Differentiated Phenotype

The production and maintenance of the terminally differentiated phenotype of striated muscle cells involves the permanent withdrawal from the cell cycle and the induction of muscle-specific genes. A common biochemical explanation for these two processes and for the mutual exclusivity between cell growth and differentiation has been lacking. Evidence recently obtained in Dr. Nadal-Ginard's laboratory indicates that central to these processes is the retinoblastoma gene product (pRB), which is required for the production and maintenance of the terminally differentiated phenotype of muscle cells. Inactivation of pRB, either through phosphorylation, binding to oncogenes, or genetic alteration, inhibits myogenesis. Moreover, inactivation in terminally differentiated cells allows them to re-enter the cell cycle. pRB is required for the myogenic and trans-activation activities of MyoD. In addition, pRB is required for the cell growth inhibitory activity of MyoD. Both *in vivo* and *in vitro*, pRB binds directly to MyoD through the pocket and the bHLH domain, respectively. This direct binding induces a family of transcription factors that operate through the MEF2 DNA-binding site, present in most, if not all, muscle-specific genes, as indicated in the previous section.

The fact that cell growth and differentiation are mutually exclusive in myogenic, as well as other

terminally differentiated cell types, is a puzzle that until now has defied a molecular explanation. The dual role of pRB in cell cycle control and muscle-specific gene transcription explains a major aspect of this apparent paradox. Unphosphorylated pRB is required for myogenesis and muscle-specific gene expression through its interaction with MyoD; specific phosphorylation of pRB is required for cell cycle progression. Thus, through its mutually exclusive interaction with cell cycle regulators and myogenic factors, pRB can function as a binary switch that determines whether the cell progresses through the cell cycle or commits to the terminally differentiated pathway.

At the basis of the model described here are the multiple interactions available to each of the main players responsible for cell differentiation and tissue-specific gene expression. This striking feature permits them to function as binary switches. In the presence of high concentrations of growth factors, each of these regulators can further stimulate cell growth; under low-growth conditions, each can serve as a cell growth suppressor and play a role in the production of the terminally differentiated phenotype. By replacing MyoD for other lineage-determining genes, the model emerging from myogenesis is likely to apply to other terminally differentiated systems.

Alternative Splicing of Contractile Protein Genes

The laboratory's mechanistic analysis of alternative splicing has concentrated on the α-tropomyosin exons 2 and 3. These exons are mutually exclusive: exon 3 is found in the mRNA of most cells (the default pathway); exon 2 is found only in the mRNA of smooth muscle (the regulated pathway). The primary determinant for the default pathway is the strength of the branch point/polypyrimidine tract upstream of these two exons. Dr. Nadal-Ginard and his colleagues previously demonstrated that the strength of a given exon in cis-competition splicing assays is directly correlated with the ability of the polypyrimidine tract–binding protein (PTB), characterized in this laboratory and in Dr. Phillip Sharp's laboratory (Massachusetts Institute of Technology), to be crosslinked to the polypyrimidine tract of the exon. The interaction of PTB with the pre-mRNA has been exploited to investigate the very early step in spliceosome assembly and to determine when splice site selection and commitment to a given splicing pattern occurs. These studies have revealed that U1 snRNP associates with the 3′ end of the intron in the earliest step of spliceosome formation in an ATP-independent manner. Since it has been

known for some time that U1 snRNP interacts with the 5' splice site, this molecule brings together the two ends of the intron.

To verify the ability of a single U1 snRNP to interact with both the 5' and 3' ends of the intron, the laboratory utilized a pre-mRNA splicing substrate cleaved by RNase H into 5' and 3' half-molecules. If the intron is cleaved after complex formation in nuclear extract lacking ATP, both halves are immunoprecipitated with U1 antisera. However, when the RNA is cleaved before incubation with extract, only the 5' half-molecule is immunoprecipitated. Since complex assembly on an intact pre-mRNA enhances precipitation of the 3' half-molecule, colinearity of the splice sites must stabilize the association of U1 with the 3' end, implying that a single U1 bridges both splice sites in ATP-independent complexes. Surprisingly, the same results are obtained when the 5' end of U1 snRNA is removed by RNase H digestion. This suggests that the ATP-dependent association between U1 snRNA with both the 5' and 3' splice sites does not require the U1 snRNA 5' end, even though this is the sequence that base pairs with the 5' splice site. Therefore the ATP-independent interaction between U1 and the splice sites is distinct from the ATP-dependent association between U1 and the 5' splice site and must be mediated by a different region of U1 and/or additional factors. Experiments are in progress to characterize this earliest step in splice site selection.

The experiments outlined above indicate that while the 5' splice site, the branch point, and polypyrimidine tract are required early in the splicing pathway and are recognized by established splicing factors, the mode of recognition of the 3' splice site AG and the stage in the pathway at which this recognition occurs remain less understood. This laboratory previously proposed that the 3' splice site in mammalian introns is identified and located by a scanning mechanism that searches for the first AG downstream from the branch point/polypyrimidine tract. During the past year experiments have been carried out to support and refine this model. In addition, prompted by examples of various natural introns, the basis for the exception to the simple rule that the first AG downstream from the branch point is selected exclusively as the 3' splice site has been explored. The results demonstrate that, although such exceptions do exist, they are predictable and can easily be accommodated by simple refinements of the model. This behavior is reminiscent of the scanning model for translational initiation, which has also been able to accommodate exceptions with simple "rules to break the rule." In light of these findings, a refined scanning model can be summarized as follows: scanning initiates at the branch point and proceeds in a 3' direction to the first AG unless it is either so close to the branch point that recognition is very inefficient or it is hidden within a stem-loop structure. In either of these cases the first AG can be bypassed, and the next downstream AG located. Once an AG has been recognized, however, the spliceosome can still "see" a limited stretch of downstream RNA. Within this "window," the most competitive AG is selected as the 3' splice site.

Determinants of competitiveness include proximity to the branch point: the more-proximal AG is usually more competitive, unless it is very close to the branch point, in which case steric effects can render it less competitive. The nucleotide preceding the AG also has a marked effect upon competition. The hierarchy of competitiveness, $CAG \cong UAG > AAG \gg GAG$, closely follows the occupancy of this position in consensus compilations of 3' splice sites. Thus 3' splice site selection displays properties of both a scanning process and competition between AGs based on immediate sequence context. Competition based on the preceding nucleotide and steric effects explains two features of consensus 3' splice site arrangements: the nucleotide preference at the -3 position and the common 18-nt minimum separation between the branch point and the 3' splice site AG.

Dr. Nadal-Ginard is also Alexander S. Nadas Professor of Pediatrics and Professor of Cellular and Molecular Physiology at Harvard Medical School and Cardiologist-in-Chief at the Children's Hospital, Boston.

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GENOMIC RESPONSE TO GROWTH FACTORS

Daniel Nathans, M.D., *Senior Investigator*

Proliferation of mammalian cells is regulated by polypeptide growth factors. Growth factors act via specific cell surface receptors and intracellular messenger systems to induce genetic programs in target cells. Research in the laboratory of Dr. Nathans has been aimed at characterizing the genetic program induced in cultured fibroblasts by serum growth factors.

When nonproliferating mouse 3T3 cells are stimulated with serum or platelet-derived growth factor (PDGF), sets of genes are activated sequentially. The first set of genes, called immediate-early genes, is activated within minutes after the cells are stimulated. They include genes that encode a variety of transcription factors, among them leucine zipper proteins (Fos and Jun family members), zinc finger proteins (Zif268 and related proteins and Nur77), and helix-loop-helix proteins (Myc, HLH462). Another set of genes, called delayed-early genes, is expressed within a few hours after cells are stimulated. Expression of delayed-early genes requires new protein synthesis, presumably the synthesis of immediate-early transcription factors responsible for activating delayed-early genes. During the past year studies have focused on selected immediate-early transcription factor genes and on systematic identification of delayed-early genes and their possible regulatory sequences.

Activation of *jun-B*

Several immediate-early genes are activated by serum or PDGF through one or more serum response elements (SREs) upstream of each gene. In the case of *jun-B*, the upstream sequence near the start of the gene has no SRE, nor is an upstream segment linked to CAT sufficient for response to activation by growth factors. By assaying a series of mutant *Jun-B* genes for transcriptional activation by serum or PDGF, Evelio Perez-Albuerne (a graduate student in Dr. Nathans' laboratory, whose research is supported by the National Cancer Institute) has found a regulatory region immediately downstream of *jun-B*. Within the regulatory region is a typical SRE and a cAMP response element (CRE). Nucleotide substitutions within the SRE reduce responsiveness of *jun-B* to serum, PDGF, and fibroblast growth factor (FGF). Nucleotide substitutions within the CRE reduce responsiveness to forskolin. Thus the mechanism of activation of *jun-B* by serum, PDGF, or FGF appears to be similar to that found for a number of other immediate-early genes, except that the response element is farther away from the start of transcription and is downstream of the gene.

Proteins That Interact with Jun and Fos

Jun and Fos family members are sequence-specific, DNA-binding transcription factors. To bind

DNA they must first form homodimers or heterodimers via coiled-coil structures made up of leucine zipper domains. Certain other leucine zipper DNA-binding proteins have also been shown to form heterodimers with Jun. To identify additional proteins that dimerize with the leucine zipper of Jun or Fos, Pierre Chevray (a graduate student in Dr. Nathans' laboratory, whose research is supported by the National Cancer Institute) has used a yeast genetic system described by Drs. Stanley Fields and Ok-kyu Song to clone mouse cDNAs encoding polypeptides that interact with the leucine zipper segment of c-Jun or c-Fos.

Several proteins were identified, some showing interaction only with Jun or only with Fos. One of the proteins is a member of the ATF/CREB family of transcription factors, probably the murine homologue of TAXREB67. Others are α- and β-tropomyosins, which are known to form coiled-coil structures via extensive leucine zipper domains, and several are previously undescribed proteins with predicted potential to form coiled coils. In addition, Dr. Timothy Schaefer (Associate in Dr. Nathans' laboratory) has used the yeast system to identify proteins that interact with Jun outside the zipper domain.

Delayed-Early Genes

To identify delayed-early genes in a systematic way, Dr. Anthony Lanahan (Associate in Dr. Nathans' laboratory) constructed a 3T3 cell cDNA library enriched in delayed-early cDNAs. As reported briefly last year, he selected by differential screening cDNAs derived from mRNAs that are induced by serum with delayed-early kinetics. About 40% of the 650 primary cDNA isolates are represented by 13 different cDNAs. Partial or complete sequencing of the 13 cDNAs carried out by Dr. Lanahan and Dr. John Williams (an HHMI Postdoctoral Research Fellow) revealed that 9 are related to known proteins and 4 are not. Among the former are cDNAs encoding the nonhistone chromosomal proteins HMGI(Y) and HMGI-C, adenine phosphoribosyltransferase (APRT), DNA polymerase δ, a protein related to human macrophage migration inhibitory factor (MIF), the murine homologue of CHIP28 (an integral membrane protein of human erythrocytes involved in water transport), and cyclin CYLl. On the as-

sumption that most of the more abundant cDNA species have already been detected, one can estimate that several dozen delayed-early cDNAs in the collection remain to be characterized. Thus the delayed-early gene response is likely to be at least as complex as the immediate-early gene response.

Each of the delayed-early mRNAs so far identified appears or increases a few hours after cell stimulation by serum, PDGF, or FGF, well before the onset of serum-induced DNA synthesis, and the response is inhibited by cycloheximide and anisomycin. In each case gene transcription is stimulated by the growth factor. For some of the genes, potential binding sites for immediate-early transcription factors have been detected upstream of the transcription start site. Studies are in progress to determine whether these genes are activated by the transcription factors.

Dr. Nathans is also University Professor of Molecular Biology and Genetics at the Johns Hopkins University School of Medicine.

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MOLECULAR BIOLOGY OF VISUAL PIGMENTS

JEREMY NATHANS, M.D., PH.D., *Associate Investigator*

Visual pigments are the light-absorbing proteins that initiate phototransduction. Each consists of an integral membrane protein covalently joined to a chromophore, 11-*cis* retinal. Light photoisomerizes retinal from 11-*cis* to all-*trans*, which in turn produces a conformational change in the attached apoprotein, converting it into an activator of a photoreceptor-specific G protein.

Dr. Nathans is investigating several questions related to visual pigment structure and function. Which residues comprise the chromophore binding site and how do they modify the photochemistry of retinal? How do visual pigment genes vary in the human population, and what are the consequences of this variation for visual function? How does each type of photoreceptor cell determine which visual pigment to express?

Color Vision and Color Blindness

Human color vision is based upon a comparison of the extent of excitation of three visual pigments that reside in three classes of cone photoreceptor cells. The nucleotide sequences of genomic and cDNA clones encoding the three human cone pigments reveal 42% amino acid identity between the blue pigment and the red or green pigments, whereas the red and green pigment sequences show 96% mutual identity.

Shannath Merbs, an M.D.-Ph.D. student, has recently succeeded in producing the human cone pigment apoproteins in transfected tissue culture cells. Following reconstitution with 11-*cis* retinal, the visual pigments show absorption maxima of 426 nm (the blue pigment), 530 nm (the green pigment), and 552 or 557 nm (two allelic variants of the red pigment). The two red pigment variants differ by an alanine-versus-serine substitution at position 180. A spectral difference of 5 nm would be predicted to produce a readily measurable perceptual difference between individuals. Indeed, Dr. Samir Deeb and his colleagues at the University of Washington have shown that this allelic variation correlates well with differences in color-matching ability between individuals with normal color vision. In the human gene pool, alanine is encoded at this position in ~40% of red pigment genes and serine in the remaining 60%.

In more recent experiments, Ms. Merbs has studied a set of hybrid pigments corresponding to those produced by homologous unequal recombination between red and green pigment genes. The hybrids are found in individuals with anomalous trichro-

macy, a variant form of color vision present in ~6% of Caucasian males. The absorption spectra of the nine most common hybrids all lie within the interval defined by the spectra of the red and green pigments from which they were derived. Amino acid differences in exon 5 appear to exert the largest effect on spectral tuning. These absorption spectra provide a framework for predicting the characteristics of the various anomalies of red-green color vision. Experiments now in progress are aimed at precisely defining the contribution to spectral tuning of each of the 15 amino acids that differ between red and green pigments. (The project described above was supported in part by a grant from the National Eye Institute, National Institutes of Health.)

Blue Cone Monochromacy

A rare form of severe color blindness called blue cone monochromacy results from mutational events at the red and green pigment gene locus that eliminate the activity of both genes. To date, 40 families with blue cone monochromacy have shown large-scale rearrangements at the red and green pigment gene cluster. One class of rearrangements defines a small DNA region adjacent to the visual pigment genes that appears to be essential for their correct expression. This region is located 3 kb from the adjacent red pigment gene and 42 kb from the nearest green pigment gene.

Yanshu Wang, a graduate student, has shown that in transgenic mice this segment is required for expression of a β-galactosidase reporter gene in cone photoreceptors. The essential region coincides with a sequence of several hundred bases that are highly conserved between humans, mice, and cattle. These experiments suggest a model in which the upstream controlling region and its associated proteins interact with either the red or green pigment gene promoter. If the controlling region can accommodate only one such interaction, this mechanism would ensure the mutually exclusive expression of red and green pigment genes in their respective cone photoreceptors.

Approximately 50% of blue cone monochromats have only a single gene in the red and green pigment gene array. Most of these single genes carry a cysteine-201–to–arginine mutation that disrupts an essential disulfide bond. One blue cone monochromat has two genes in his visual pigment array, both of which carry this same mutation. Curiously, several blue cone monochromats have visual pigment

gene arrays in which the nucleotide sequences of the coding regions and promoter are normal.

Retinitis Pigmentosa

Over the past three years, Dr. Ching-Hwa Sung, Ms. Jennifer Macke, and Dr. Nathans have looked for naturally occurring mutations in the gene encoding human rhodopsin. Rhodopsin mediates vision in dim light and resides in the photoreceptors of the peripheral retina. Deficiencies in night and peripheral vision are the hallmarks of retinitis pigmentosa (RP), a heterogenous group of inherited progressive retinal dystrophies. In an initial survey, 13 rhodopsin point mutations were identified in 39 of 161 families with autosomal dominant RP, and in each case the mutation was found to co-inherit with retinal disease. Five new mutations have recently been identified, and together with those found by other investigators, the total number of rhodopsin mutations found among RP patients is now over 40.

To examine the biochemical properties of the defective rhodopsins, Dr. Sung, Ms. Carol Davenport, and Dr. Nathans have produced 34 of them in tissue culture cells. Most of the mutant rhodopsins appear to be defective in their folding and/or stability. These proteins accumulate in the endoplasmic reticulum and are degraded more rapidly than the wild type.

A subset of the mutations cluster near rhodopsin's carboxyl terminus. These proteins fold correctly and are transported to the plasma membrane. The nature of their biochemical defect is currently under investigation. (The project described above was supported in part by a grant from the National Retinitis Pigmentosa Foundation.)

Dr. Nathans is also Associate Professor of Molecular Biology and Genetics and of Neuroscience at the Johns Hopkins University School of Medicine.

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GENE REGULATION IN ANIMAL CELLS

Joseph R. Nevins, Ph.D., *Investigator*

The research in Dr. Nevins's laboratory is focused on three aspects of gene regulation in animal cells: 1) the mechanism of action of viral oncoproteins, 2) use of viral systems to elucidate mechanisms of transcriptional regulation, and 3) molecular mechanisms of regulation of RNA processing at polyadenylation sites.

Molecular Mechanisms for Oncogenesis by Viral Proteins

In 1988, Dr. Ed Harlow and his colleagues demonstrated that the adenovirus E1A oncoprotein is able to bind to the retinoblastoma-susceptibility gene product (Rb) and that this interaction is dependent on E1A sequences that are essential for oncogenic activity. This finding, and the subsequent realization that the interaction was also a property of the oncoproteins of the other DNA tumor viruses, such as simian virus 40 (SV40) T antigen, and human papillomavirus (HPV) E7, strongly suggested that these viral proteins elicited cellular transformation by inactivating the Rb tumor-suppressor protein. Dr. Nevins and his colleagues have now demonstrated that the cellular transcription factor E2F is a target for control by the Rb tumor-suppressor protein and that the interaction of the viral oncoproteins with Rb disrupts this control.

Moreover, the relevance of these events to human cancer is indicated by Dr. Nevins's findings that the E2F-Rb interaction is absent in various human cervical carcinoma cell lines that either express the HPV E7 protein or that harbor an inactivating mutation in the *RB1* gene, suggesting that the loss of the E2F-Rb interaction is an important aspect of human cervical carcinogenesis. It thus appears that the ability of E1A, SV40 T antigen, and HPV E7 to dissociate the E2F-Rb complex is a common activity of these viral proteins that has evolved to stimulate quiescent cells into a proliferating state.

Further analyses of the interaction of E2F with regulatory proteins such as Rb have shown that the transcriptional activity of E2F is inhibited. Since E2F likely controls the transcription of cellular genes that are important for S-phase events, this control appears to be an integral part of cellular proliferation regulation. In this regard, an additional E2F interaction has been identified that is regulated at the transition from G1 to S phase. This complex contains the Rb-related p107 protein in association with E2F. Moreover, like the Rb protein, p107 in-

hibits E2F-dependent transcription in a cotransfection assay. This result, together with the observation that free, uncomplexed E2F accumulates as cells leave G1 and enter S phase, suggests that the p107 protein may regulate E2F-dependent transcription during G1.

Mechanisms of Transcriptional Regulation in Virus-infected Cells

Complex cellular events are often simplified through the use of model systems provided by viruses that infect these cells. The mediation of transcription control by the adenovirus E1A gene product has proved valuable in deciphering mechanisms that control transcription in animal cells.

Recent studies have shown that the adenovirus $E1A_{12S}$ protein (the oncogenic E1A product) can trans-activate transcription by releasing the E2F transcription factor from inhibitory complexes with proteins such as Rb. However, E2F cannot be the only target for E1A activation, since several cellular promoters have been found to be activated by the E1A protein despite the fact that they lack E2F sites. Indeed, activation of the hsp70 promoter by E1A requires the TATAA sequence. Moreover, E1A protein domains that are required for activation of E2 transcription via E2F are not required for activation of the hsp70 promoter. Rather, a distinct protein domain facilitates TATAA-dependent transcriptional activation. It appears that the targeting of distinct transcription factors, leading to trans-activation of transcription of multiple promoters, involves distinct domains of the E1A proteins that are also required for oncogenic activity.

Given the fact that the TATAA element is a target for the activation of the hsp70 promoter, Dr. Nevins and his colleagues have addressed the possibility that interactions with the TATAA-binding protein (TBP) might be altered by E1A. Recent experiments in the laboratory of Dr. Danny Reinberg and his colleagues have identified an activity termed Dr1 that interacts with and inhibits the transcriptional activity of TBP. Dr. Nevins's work now demonstrates that the E1A protein can disrupt the interaction of Dr1 with TBP, allowing TBP to then interact with the TFIIA transcription factor. This disruption depends on the amino-terminal E1A sequences that are also essential for trans-activation of the hsp70 promoter. It would thus appear that the activation of hsp70 through the TATAA element may be mechanistically

similar to the activation of the E2 promoter via E2F, in each case involving a release of the responsible transcription factor from an inactive complex.

Molecular Mechanisms of Polyadenylation

The generation of the 3' terminus of the mature mRNA, commonly termed poly(A) site formation, is a critical event in the biogenesis of most mRNA molecules. Moreover, since many transcription units encode multiple mRNAs that utilize alternative poly(A) sites, there is a potential regulatory role for polyadenylation. For instance, alternative poly(A) site choice is a contributing factor in the switch from production of the immunoglobulin heavy-chain mRNA that encodes the membrane-bound form of the protein to the mRNA encoding the secreted form of the protein. Thus an understanding of the mechanisms of poly(A) site utilization, including the sequences directing the processing event as well as the factors involved in processing, is of considerable importance.

One additional example of poly(A) site regulation is found during the course of an adenovirus infection. Five poly(A) sites are utilized within the major late transcription unit to produce more than 20 distinct mRNAs during the late phase of infection. The proximal half of the major late transcription unit is also expressed during the early phase of a viral infection. During this phase, the L1 poly(A) site is used three times as frequently as the L3 poly(A) site. In contrast, the L3 site is used three times more frequently than L1 during the late phase of infection. Recent experiments have suggested that the recognition of the poly(A) site GU-rich downstream element by the CF1-processing factor may be a rate-determining step in poly(A) site selection.

Work in Dr. Nevins's laboratory has demonstrated that the interaction of CF1 with the L1 poly(A) site is less stable than its interaction with the L3 poly(A) site. Moreover, there is a substantial decrease in the level of CF1 activity when an adenovirus infection proceeds to the late phase, suggesting that this reduction in the CF1 activity, coupled with the relative instability of the interaction with the L1 poly(A) site, contributes to the reduced use of the L1 poly(A) site in a late-infected cell.

Dr. Nevins is also Professor of Genetics at Duke University Medical Center.

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MOLECULAR GENETICS OF X-LINKED DISEASE

Robert L. Nussbaum, M.D., *Associate Investigator*

Dr. Nussbaum's laboratory has been engaged in positional cloning efforts directed toward identifying X-linked genes responsible for human genetic disease. Three disorders have been the targets of these efforts: fragile X syndrome, Lowe oculocerebrorenal syndrome, and choroideremia. As a result of the efforts of a number of laboratories around the world, including Dr. Nussbaum's, all three of these genes have been identified. During the past year Dr. Nussbaum's laboratory has shifted its efforts from positional cloning to a detailed genetic analysis of the biological basis of one of these diseases, the Lowe oculocerebrorenal syndrome (OCRL), and the complex area of the metabolism that his research indicates is affected by this disease.

OCRL is an X-linked disorder characterized by mental retardation, congenital cataracts, renal tubular dysfunction in childhood, and progressive renal failure in adulthood. Dr. Nussbaum's laboratory used genomic cloning in the vicinity of two X chromosome–autosome translocations in two females with OCRL to identify the *OCRL-1* gene, which is interrupted by both translocations. Northern blot analysis revealed that the mRNA for this gene is absent in both female patients and is missing or abnormal in size in 9 of 13 unrelated male patients with the disorder. This suggests that *OCRL-1* is a reasonable candidate for the gene involved in the disease. (The preliminary work that formed the basis for the isolation of *OCRL-1* was supported by a grant from the National Institute of Child Health and Development, National Institutes of Health.)

The predicted amino acid sequence for *OCRL-1* is consistent with a 970–amino acid protein (theoretical molecular weight 112 kDa). Sequence analysis showed that *OCRL-1* is very similar but not identical to a previously described cDNA (HUMIN5P5), which encodes an enzyme, type III inositol polyphosphate-5-phosphatase, that removes the phosphate at the 5 position from inositol-1,4,5-trisphosphate. Inositol is a six-carbon ring sugar alcohol with a complex cellular metabolism that has been the subject of intensive biochemical investigation. Inositol phosphate esterified to diacylglycerol (phosphatidylinositol) makes up 2–8% of cellular phospholipid. It undergoes a complex cycle of phosphorylation, dephosphorylation, phospholipase cleavage, and re-esterification. Inositol phosphate is clearly involved in signal transduction and in anchoring membrane proteins by glypiation, but

(as is the case with many biochemical investigations) the physiological functions of most inositol phosphates, either free or esterified in phospholipids, are incompletely understood. The results in Dr. Nussbaum's laboratory suggest that OCRL may be the first human inborn error of inositol phosphate metabolism and that at least one disease phenotype can result from a defect in its metabolism.

Strong evidence that *OCRL-1* is the genetic locus for OCRL has been obtained by demonstrating single-point mutations in the *OCRL-1* gene from severely affected OCRL patients. These mutations disrupt mRNA processing and translation and lead to premature translation termination and loss of the carboxyl-terminal 25–30 amino acids. This region of the protein is likely to be important for the function of the enzyme, because it is highly conserved between the *OCRL-1* protein and HUMIN5P5.

The next goal is to define the mechanism by which this defect in inositol metabolism disrupts lens development and interferes with normal renal and neurological function. Because such studies cannot be undertaken in humans, the laboratory has begun to study the role of the *OCRL-1* gene product in frogs and mice. Embryonal stem cell technology is being used to create mouse models for OCRL. The mouse homologue for *OCRL-1* has been isolated, and the exons encoding the terminal 100 amino acids of the protein have been isolated and sequenced. DNA constructs have been made that will be used to recombine with the normal mouse gene and cause a deletion of exons encoding the carboxyl terminus of the protein, because human mutations that truncate the terminal 20–30 amino acids of the *OCRL-1* protein cause severe disease. The *Xenopus* homologue for *OCRL-1* has also been isolated. The timing and tissue distribution of expression of this gene during tadpole development is being studied, with emphasis on its role in the development of the amphibian lens.

The *OCRL-1* gene product is only one of a myriad of enzymes implicated in inositol phosphate metabolism. Because it is difficult to assess the functional significance of all these enzymatic activities, the laboratory is beginning a systematic program to isolate and delete other genes involved in inositol metabolism in embryonal stem cells to create mice deficient in these enzymatic activities. In this way the functional significance of these metabolites and the physiological role of

the enzymes responsible for creating and metabolizing them can be assessed.

As a by-product of the studies directed toward isolating the OCRL gene, Dr. Nussbaum and his colleagues found, entirely by chance, the human gene *hSNF2L*. Although not involved in OCRL, this gene has interesting homologies to a number of previously identified genes in yeast and *Drosophila*. *SNF2/SWI2* and *STH1* from *Saccharomyces cerevisiae* and *brahma* in *Drosophila melanogaster* represent a class of transcription factors or cofactors termed "global" activators of transcription. The Snf2 and brahma proteins appear to exert their effects on transcription as part of multifactor complexes and may serve as bridges between specific DNA-binding proteins and the transcriptional apparatus. The strong similarity between *hSNF2L* and these yeast and *Drosophila* genes suggests that the mammalian gene is part of an evolutionarily conserved family that has been implicated as global activators of transcription in yeast and fruit flies but whose function in vertebrates is unknown. The laboratory has begun to isolate and characterize the murine and amphibian homologues of *hSNF2L*, in order to study the timing and tissue distribution of its expression and to create a deletion mutation of this gene in mice.

Dr. Nussbaum is also Associate Professor of Human Genetics, Pediatrics, and Internal Medicine at the University of Pennsylvania School of Medicine and Consultant in Genetics at the Children's Hospital of Philadelphia.

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ANALYSIS OF THE YEAST AND HUMAN GENOMES

Maynard V. Olson, Ph.D., *Investigator*

Dr. Olson's laboratory is pursuing interrelated studies of the yeast and human genomes, with the long-range goal of defining the structure and function of the chromosomal DNA of these organisms. This research has three objectives: 1) to produce detailed physical maps of yeast and human DNA, which will facilitate molecular genetic studies of these organisms; 2) to advance the associated methodology, which will benefit research on all higher organisms; and 3) to study the functional behavior of large genes, gene clusters, and other segments of mammalian chromosomes.

The Yeast Genome

The yeast genome is ~15 million base pairs (bp) in size. Depending on the genetic background of the yeast strain, 1 or 2 million bp represent rDNA (i.e., they encode RNA components of the ribosomes), while the remaining 12.5 million bp encompass the rest of the nuclear genes. Dr. Olson's laboratory has developed detailed physical maps of this DNA that show the positions of known genes relative to DNA landmarks, such as chromosome ends and sites at which particular site-specific restriction endonucleases cleave the DNA.

More than 5,300 bacteriophage λ and cosmid clones of yeast DNA have been analyzed by computer-based techniques and used to construct contigs (sets of overlapping clones that cover a contiguous region of the genome). These contigs now cover >95% of the non-rDNA component of the yeast genome. Most of the missing DNA is at the extreme ends of the chromosomes, which are inaccessible to standard cloning techniques. Much of the effort during the past year has been directed toward closure of the contig maps, the process of analyzing and then eliminating gaps between contigs. This process becomes progressively more difficult as the number of gaps decreases, since the gaps that remain are those that have resisted analysis by standard methods. Since the last annual report the number of internal gaps has been reduced from 69 to 12, with a corresponding increase in average contig size from 160 to 411 kbp.

The contig maps facilitate the localization of newly cloned yeast genes relative to previously defined genes. To aid in this process, 186 genes that have been mapped genetically through the analysis of genetic recombination during sexual crosses have also been localized on the physical maps. New genes can be localized relative to these previously

defined genes by DNA-DNA hybridization assays that identify the mapped clones that contain the new gene. These assays take only a few hours and employ a set of three small nylon filters produced by Dr. Olson's laboratory. During the past year the number of laboratories using these filters has increased from 70 to 150. This convenient and reliable style of gene mapping is becoming an early step in the characterization of a yeast gene. Early mapping allows a quick determination of whether the gene has been studied previously, thereby saving much duplication of effort in yeast molecular genetics.

Physical Mapping of Human Chromosomes

The average human chromosome contains 10 times as much DNA as the entire yeast genome. Furthermore, there have been serious difficulties in obtaining continuous cloned coverage of human chromosomes in clones that are propagated in *Escherichia coli*. Consequently, a direct extension to human chromosomes of the methods employed to map the yeast genome would be unlikely to succeed.

As an alternative strategy, Dr. Olson's laboratory has been pursuing applications of the yeast artificial chromosome (YAC) system to the physical mapping of human chromosomes. YAC vectors allow large segments of exogenous DNA to be propagated in yeast as linear, artificial chromosomes. During recent years, Dr. Olson's laboratory has developed methods for constructing large libraries of YAC clones containing human DNA, for identifying specific human genes in these libraries, and for analyzing individual clones. Particularly effective has been the use of sequence-tagged sites (STSs) as mapping landmarks. STSs, which are based on random tracts of DNA sequence, can be recognized using the polymerase chain reaction (PCR). A major advantage of STS-based physical maps is that they can be described in a database in sufficient detail to allow any laboratory access to the mapped DNA without the need for supporting archives of biological materials.

A method of constructing YAC contigs, which has been named STS-content mapping, has been developed. In this method, overlaps between clones are recognized by shared STS content. Several genome centers involved in the international effort to map the human genome have adopted STS-content mapping as the first-stage method of carrying out large-scale physical mapping.

Attention in Dr. Olson's laboratory is now shifting

to the second-stage problem of mapping multi-mega-base pair contigs in sufficient detail to support large-scale DNA sequencing. This application requires the mapping of enormous numbers of sites. Even a 1-Mbp region, mapped at a resolution of 100 bp, requires the mapping of 10,000 sites. Furthermore, the need to solve such problems repeatedly requires an automated strategy. Emphasis is presently on the computational problems posed by automated high-resolution mapping projects. Traditionally computers have been used as little more than bookkeeping tools in physical mapping projects; most substantive decisions have been made by a human analyst. However, the scale of the mapping problems posed by the human genome requires that interactive human involvement in the data analysis be minimized.

Functional Studies

YACs also provide a path toward the functional analysis of large segments of the chromosomes of higher organisms. The experimental approach involves cloning the segments into yeast and then transferring the YACs into mammalian cells where their genetic effects can be evaluated. Test systems involve the human HPRT (hypoxanthine phosphoribosyltransferase) and GART (phosphoribosylglycinamide formyltransferase) genes, both of which encode enzymes required for the *de novo* synthesis of purines in mammalian cells. Both human genes have been cloned in YACs and transferred to mutant rodent cells that lack HPRT and GART activities. The cloned genes have proved to be fully functional in the rodent cells to which they were transferred.

Past successes in transferring YACs to mammalian cells have largely relied on fusing mammalian cells with yeast cells whose cell walls have been removed. Although effective in some cases, cell fusion fails with many mammalian cells. A more robust procedure is microinjection of the YAC DNA. The fragility of large DNA molecules has required the development of specialized handling procedures to allow microinjection of intact YACs. During the past year, major improvements in such procedures have allowed intact DNA segments as long as 500 kbp to be transferred to mammalian cells by microinjection.

Dr. Olson is also Professor of Genetics at Washington University School of Medicine, St. Louis. Effective September 1, 1992, he will assume the position of Professor of Molecular Biotechnology at the University of Washington, Seattle.

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GENETIC HEMATOLOGY

Stuart H. Orkin, M.D., *Investigator*

Hematopoietic cells develop from pluripotent stem cells in the bone marrow. During their differentiation, they progressively commit to specific lineages, such as red, white, and lymphoid cells, and the cells of each lineage synthesize characteristic gene products. The efforts of this laboratory are directed toward an understanding of the molecular basis of cell commitment and of the mechanisms that regulate coordinated expression of genes in developing blood cells. Findings derived from these studies will provide further understanding of cellular development in vertebrates and may bear on the

pathophysiology and management of inherited and acquired disorders of hematopoiesis.

Erythroid Cell Development

During red blood cell development, globin genes are activated and precisely regulated, both with respect to cell lineage and temporal expression of individual members. These genes are expressed in a developmental sequence from embryonic through adult life. To address molecular mechanisms of red cell development, Dr. Orkin and his colleagues have focused on a DNA-binding protein, GATA-1, that participates in transcriptional control of all known erythroid-expressed genes.

This protein, which recognizes a consensus GATA motif in its DNA targets, is part of a larger family of proteins that are related by virtue of highly similar "finger" DNA-binding domains. GATA-1 is expressed at the very earliest stages of blood cell development and in multipotential progenitor hematopoietic cells that have not yet chosen a unique development path.

Previously, gene-targeting experiments in mouse embryo-derived stem (ES) cells established an essential role for GATA-1 in erythroid development. More recently, a stringent assay for GATA-1 function *in vivo* has been devised that employs introduction of the GATA-1 gene into GATA-1⁻ ES cells to rescue erythroid development. This assay permits delineation of the specific domains of GATA-1 required for promoting erythroid gene expression within developing cells.

In complementary studies, the binding of proteins to the critical regulatory elements of the human globin gene clusters, the locus control regions, has been evaluated by an improved *in vivo* footprinting method. These studies define GATA-1 and a second erythroid protein, NF-E2, as the critical components for establishing erythroid-specific gene expression. This latter protein, recently purified, and its cDNA cloned, will be the focus of future studies.

White Blood Cell Development

Phagocytic cells (neutrophils and macrophages) produce superoxide via an NADPH-oxidase complex as part of a major bactericidal host-defense system. Deficiency of this oxidase leads to an immune condition, chronic granulomatous disease (CGD). Previously the gene encoding a major component of the oxidase (gp91-phox) was identified through positional cloning in the laboratory. Subsequent studies have shown that mutations in the gp91-phox gene lead to one form of CGD. The gp91-phox gene is expressed only in developing phagocytic cells. To identify important regulators of white cell gene ex-

pression and development, the gp91-phox gene and its controlling elements are under study.

In transgenic mice the promoter region alone directs limited cell specificity and can target malignancy to a subset of macrophages when linked to the simian virus 40 (SV40) T antigen. Protein-binding and functional studies revealed that the gp91-phox promoter is under negative regulation through a putative repressor protein CCAAT displacement factor (CDP). Molecular cloning revealed that CDP is the counterpart of a *Drosophila* homeodomain protein, encoded by the *cut* gene, which is involved in cell-fate decisions in several tissues in the fly.

Experiments under way are aimed at determining how CDP recognizes DNA and functions to repress gene expression in undifferentiated white cells. Other experiments are aimed at defining the positive regulatory elements specifying gp91-phox expression in white cells.

Dr. Orkin is also Leland Fikes Professor of Pediatric Medicine at Harvard Medical School and the Children's Hospital, Boston.

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SPECIFICATION OF LEFT-RIGHT POLARITY

Paul A. Overbeek, Ph.D., M.B.A., *Assistant Investigator*

During mammalian development an initially symmetrical embryo proceeds to establish dorsal-ventral, anterior-posterior, and left-right polarities. Left-right asymmetries are late-appearing and are most apparent in the visceral organs such as stomach, spleen, liver, and heart. The molecular mechanisms that specify mammalian embryonic axes remain to be elucidated. Recently a transgenic family of mice that has an insertional mutation causing a reversal of embryonic left-right polarity was identified. Initial characterization of this new mutation has been pursued over the past year.

The mutation was identified in the transgenic family OVE210, which was generated by microinjection of a tyrosinase minigene into one-cell embryos of the albino mouse strain FVB/N. The albinism of non-transgenic FVB mice is due to a point mutation in the gene encoding tyrosinase, the first enzyme in the pathway to melanin synthesis. Introduction of a functional tyrosinase minigene into the FVB genome results in gene cure and restoration of the ability to synthesize melanin. Transgenic mice become pigmented and can be identified by simple visual inspection. In family OVE210 the transgenic mice have light brown fur and darkly pigmented eyes.

They have a single site of integration with duplicate head-to-tail copies of the tyrosinase minigene.

When OVE210 mice were inbred, all of the homozygotes were found to have a reversal of their visceral left-right polarity. All of the major internal organs, including heart, lungs, stomach, spleen, and pancreas, were located on the side of the body opposite their normal position. This condition also occurs occasionally in humans and is referred to as *situs inversus*.

The inbred mice, in addition to the *situs inversus*, develop severe jaundice within 24 hours after birth. Serum bilirubin levels are elevated 20-fold. The mice suckle normally but show a marked failure to thrive, typically dying 3–7 days after birth, generally at their birth weight. Examination of the visceral organs by histology revealed that the *situs inversus* mutants suffer from severe kidney pathology, with defective glomeruli and distended renal tubules.

Situs inversus in humans is often associated with structural defects in the dynein arms of the cilia (a condition termed Kartagener's syndrome). The *situs inversus* transgenic mutants were checked for ciliary defects. Ciliated tracheae were collected and

examined by electron microscopy (in collaboration with Dr. William Brinkley), but no dynein defects were detected.

Mouse geneticists had previously identified the mutation *situs inversus viscerum* (or *iv*), which maps to mouse chromosome 12. Mice that are homozygous for the *iv* mutation exhibit a random specification of left-right polarity. Fifty percent of the mutants show normal polarity, the other 50% *situs inversus*. By contrast the homozygous OVE210 mice exhibit 100% *situs inversus*. To test for possible allelism between the two mutations, homozygous *iv* mice (strain SI/Col from Dr. Robert Collins, Jackson Laboratory) were mated to heterozygous OVE210 mice. The transgenic offspring all showed normal left-right polarity, indicating that the two mutations were not allelic and suggesting that the transgenic insert inactivates a previously unidentified gene essential for the normal specification of left-right polarity.

A λEMBL3 genomic library was generated using OVE210 DNA, and four overlapping clones of the transgenic integration site were isolated. One of the clones contained the entire transgenic insert flanked on both sides by genomic sequences. These sequences have been used to confirm by restriction fragment length polymorphism (RFLP) analysis that the *situs inversus* mutants are homozygous for the transgenic DNA.

The flanking sequence probes have also been used to identify homozygous embryos for studies of early embryonic development. These studies have revealed that the *situs inversus* embryos rotate in the opposite direction from normal embryos during early development. The direction of embryonic rotation is established even before folding of the cardiac tube, which was previously assumed to represent the first step in the establishment of left-right asymmetry during mammalian development.

The flanking probes have recently been mapped onto the mouse genome in collaboration with Drs. Neal Copeland and Nancy Jenkins. Both of the probes map to the proximal region of mouse chromosome 4. Two candidate genes that had previously been mapped to the same region were *Pax-5* (a paired-box gene) and *Ggtb* (a glycoprotein galactosyltransferase). Probes for these genes were obtained from Drs. Peter Gruss and Gregory Hollis, respectively. The homozygous OVE210 mice showed no changes by RFLP analysis for either gene.

The probes flanking the transgenic insert map ~10 cm apart on mouse chromosome 4, suggesting the possibility of a large deletion or a rearrangement at the site of the transgenic insert. Two other genes had previously been mapped to the region of chromosome 4 that lies between the transgenic flanking sequences. These genes (*Tal-2* from Dr. Richard Baer; *Gt4-2* from Dr. Alexandra Joyner, HHMI International Research Scholar) showed normal patterns of hybridization to OVE210 DNA, indicating that an intrachromosomal inversion accompanies the integration of the transgenic DNA. Portions of the genomic sequences flanking the transgenic insert have been subcloned and sequenced. The sequences are not identical to any previously reported in the computer database.

In order to test for epistatic interaction between the *iv* and transgenic mutations, mating studies were performed to generate mice that were homozygous for both mutations (*iv/iv, 210/210*). The double mutants show random determination of left-right polarity, indicating that the OVE210 gene may function earlier than *iv* in the cascade that determines left-right polarity. Inactivation of *iv* randomizes the polarity regardless of the status of OVE210.

The double-mutant mice still exhibit jaundice and a failure to thrive, suggesting that the transgenic integration may have inactivated more than just one essential gene. Nonetheless, the new *situs inversus* mutation may allow the first positional cloning of a gene that specifies normal mammalian polarity.

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THE X AND Y CHROMOSOMES IN MAMMALIAN DEVELOPMENT

DAVID C. PAGE, M.D., *Assistant Investigator*

Dr. Page's laboratory has explored the structure and function of the mammalian genome and its role in embryonic development. Efforts during the past year were focused on the mammalian sex chromosomes and were particularly directed toward comprehensive mapping of the human Y chromosome, identification and characterization of genes and proteins involved in Turner syndrome, and investigation of sex determination.

Physical Mapping of the Human Y Chromosome

Complete physical maps, consisting of overlapping recombinant DNA clones spanning an entire genome, constitute powerful tools for exploring the organization of an organism's genetic material and the information it encodes. With the advent of yeast artificial chromosome (YAC) vectors, it has become feasible to consider constructing such a physical map of the human genome. The Y chromosome is a particularly appropriate target for physical mapping at this time. First, genetic mapping is impossible except in the small pseudoautosomal region, the only part of the Y chromosome to undergo meiotic recombination. Second, the Y is among the smallest of human chromosomes, its euchromatic region having an estimated size of 30 Mbp.

Dr. Page's laboratory constructed an essentially complete, low-resolution map of the Y chromosome by assembling 196 YAC clones, each containing a segment of the human Y chromosome, into a single overlapping array. This array encompasses >98% of the euchromatic portion of the chromosome. First, a library of large-insert YAC clones was prepared using genomic DNA from a human XYYYY male. The map was constructed by screening the library to identify the clones containing 160 STSs (sequence-tagged sites: short stretches of genomic sequence detected by polymerase chain reaction [PCR] assays). In all, 207 Y-DNA loci were assigned to 127 ordered intervals based on their presence or absence in the YACs, yielding ordered landmarks at an average spacing of 220 kbp across the euchromatic region. At present this is the highest resolution map of any mammalian chromosome. The map reveals that Y-chromosomal genes are scattered among a patchwork of X-homologous, Y-specific repetitive, and single-copy DNA sequences. This map of overlapping clones and ordered, densely spaced markers should facilitate complete definition of the gene content of the Y chromosome. In addition, the methods and strategy used to construct this map should be widely applicable to other human chromosomes.

Identification of Y-linked genes has historically relied on analysis of naturally occurring deletions. Such deletion mapping of the Y chromosome is practical because individuals with deletions of portions of the chromosome are viable and occur at a reasonable frequency in the population. Such individuals include XX males, some XY females, and persons in whom chromosome banding has revealed translocated, isodicentric, or ring Y chromosomes. Because the Y is a haploid chromosome, the presence or absence of Y-specific sequences in the DNA of these individuals can be assessed directly.

Having assembled a large battery of Y-DNA probes, Dr. Page and his colleagues characterized about 150 individuals with partial Y chromosomes. On the basis of these results, the euchromatic region

(short arm, centromere, and proximal long arm) of the Y chromosome was resolved into 43 ordered intervals, all defined by naturally occurring chromosomal breakpoints and averaging <800 kbp in length.

This deletion map provides a foundation for understanding the Y chromosome's role in sex determination and Turner syndrome (see below), and it should speed ongoing efforts to identify Y-chromosomal genes involved in gonadoblastoma (a rare gonadal neoplasm), male fertility, and stature. The map is now being used to pursue a poorly understood Y-linked gene encoding or regulating H-Y, a minor histocompatibility antigen. In collaboration with Dr. Elizabeth Simpson (Medical Research Council, London), the gene was localized to a small interval on the long arm of the chromosome by H-Y typing of individuals with partial Y chromosomes. (These studies of the human Y chromosome were supported by the National Institutes of Health.)

Ribosomal Protein S4 and the Molecular Genetics of Turner Syndrome

Turner syndrome is a complex human phenotype characterized by low viability *in utero*, short stature, gonadal degeneration, and various somatic anatomic defects. Often associated with an XO karyotype, Turner syndrome is one of the most common chromosomal disorders in humans. Evidence suggests that Turner syndrome is the result of haploidy for one or more genes common to the X and Y chromosomes. By deletion analysis, a 90-kbp portion of the Y chromosome was identified as the probable location of one or more Turner genes.

One gene, *RPS4Y*, has been identified within this region. A closely related gene, *RPS4X*, is located on the X chromosome. *RPS4Y* and *RPS4X* diverged from a single common ancestral gene during evolution, retain very similar intron/exon structures, and encode isoforms of ribosomal protein S4. Ribosomes from human male tissues contain both the X- and Y-encoded protein isoforms, which appear to function interchangeably. *RPS4X* is the only gene on the long arm of the human X chromosome known to escape X inactivation. It is possible that the Turner phenotype results, in part, from reduced protein synthetic capacity in embryos with one rather than two *RPS4* genes per cell.

Although the mouse X chromosome carries a homologue of human *RPS4X*, this mouse X gene is subject to X inactivation, and no homologue is found on the mouse Y chromosome. These findings may account for the less severe phenotype observed in XO mice as compared with XO humans. In collaboration with Dr. Ruth Lehmann (HHMI, Massachusetts Institute of Technology and the Whitehead Institute), Dr. Page and his colleagues have set out to examine the effects of *RPS4* deficiency on embryonic development in *Drosophila*.

Molecular Genetics of Sex Determination in Humans

By examination of Y-DNA sequences in human XX males and XY females, Dr. Page's laboratory found that whether a human embryo develops as a male or female is determined by the presence or absence of a 280-kbp region (interval 1A) constituting <0.5% of the Y chromosome. The vast majority of XX individuals with descended testes and normal male external genitalia carry the entirety of interval 1A of the Y chromosome, while 11 unrelated XY females in the Page laboratory's studies entirely lack interval 1A. Colleagues in other laboratories have identified a gene, *SRY*, that is located in interval 1A and plays a pivotal role in sex determination. Detailed studies of a critical subset of human XX males, XY females, and XY hermaphrodites confirm the sex-determining role of *SRY* and suggest that a second gene in interval 1A might also function in sex determination.

Most human XX males carry terminal portions of the short arm of the Y chromosome, while some XY females have deletions of terminal portions of the short arm of the Y. Genetic studies suggest that XX males and XY females with Y deletions are the results of reciprocal processes, all involving anomalous exchanges between the X and Y short arms. Some such events involve grossly unequal exchanges between misaligned X and Y chromosomes. Progress has been made in cloning and sequencing of the Y chromosomal breakpoints—the X-Y interchange junctions—from several XX males and XY females.

The sex-determining region of the Y chromosome, including *SRY*, is deleted in some human XY females. However, most human XY females carry Y chromosomes that, as judged by Southern blotting, appear to be grossly intact. Seventy-four such unexplained human XY females or hermaphrodites were identified, and *SRY* point mutations were found in six cases. The remaining 68 unexplained XY females and hermaphrodites may prove to carry mutations in other sex-determining genes. (These studies of sex determination were supported by the National Institutes of Health.)

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TRANSGENIC APPROACHES FOR STUDYING DEVELOPMENT

Richard D. Palmiter, Ph.D., *Investigator*

During the past year this laboratory has continued a productive collaboration with Dr. Ralph Brinster's laboratory at the University of Pennsylvania. Most projects involve introduction of foreign genes into the germline of mice as a means of studying the effects on development. These studies are currently directed toward germ cell development, neuronal development, and metallothionein gene regulation. This report covers the latter two areas. The work is supported in part by grants from the National Institutes of Health.

Neuronal Development

A major goal of this research is to study the development of neurons that use catecholamines as neurotransmitters. To gain experimental access to these neurons, the genes involved in catecholamine biosynthesis were cloned. Their regulatory regions are then used to direct the expression of reporter genes (e.g., the *Escherichia coli lacZ* gene) to neurons, which provides a facile means of identifying the catecholaminergic neurons in whole embryos or histological sections. This analysis was started with the human dopamine β-hydroxylase (DBH) gene, which was shown to direct expression of *lacZ* to noradrenergic neurons and chromaffin cells. More recently the 5'-flanking regions of the mouse phenylethanolamine *N*-methyltransferase (PNMT) gene was shown to direct *lacZ* expression to adrenergic neurons and chromaffin cells.

Having demonstrated that the 5'-flanking regions of these genes can direct appropriate expression of reporter genes, the next step was to use those sequences to direct the expression of other genes that might influence neuronal development. The most extreme examples of this approach are experiments in which the 5'-flanking region of the PNMT gene was used to direct the expression of diphtheria toxin to cells that normally would express PNMT. The consequence was the ablation of adrenal chromaffin cells, some retinal cells, and the adrenergic neurons of the brainstem. As expected, there was no PNMT activity and no epinephrine in these mice. When they were crossed with mice expressing PNMT-lacZ, there was no β-galactosidase activity. These mice now provide a model system in which to

examine the significance of epinephrine as an adrenal hormone and the function of adrenergic neurons in the central nervous system.

The DBH gene promoter has been tested with genes encoding a variety of interesting molecules to ascertain whether and how the promoter-gene combination might influence development of the sympathetic nervous system. Experiments are under way to test the expression of growth factors (e.g., nerve growth factor, NGF; fibroblast growth factor, FGF; and stem cell factor, SCF), oncogenes (e.g., *myc*, *ras*, and *fos*), and proteases (e.g., plasminogen activator and stromelysin).

For example, the promoter and NGF resulted in sympathetic ganglia that were much larger than normal and had more neurons than controls. The sympathetic nerve fibers were also larger than normal. They generally appeared to take normal routes to peripheral target tissues, although some nerves grew inappropriately into the adrenal gland, vagus nerve, and dorsal spinal roots. Despite the increased number of neurons reaching peripheral tissues, innervation within the tissue was decreased, as judged by catecholamine histofluorescence, neurofilament immunoreactivity, or catecholamine uptake. These observations suggest that NGF gradients are not required to guide sympathetic neurons to targets but are required for establishment of the normal density of innervation within the target tissue.

Another experimental goal is to change genetically the neurotransmitters that sympathetic neurons make. In the first experiment of this type, the DBH promoter was fused to the structural gene for PNMT with the aim of converting noradrenergic neurons to adrenergic neurons. Experimentally the strategy worked well. Sympathetic neurons had more PNMT activity than is normally present in the adrenal gland, and epinephrine levels were high in sympathetic ganglia and their peripheral targets. However, the elevated levels of epinephrine were not accompanied by a significant decrease in norepinephrine. Thus this experiment resulted in noradrenergic neurons that also make epinephrine, rather than a complete conversion. Considering that the neuronal PNMT activity was very high, the results suggest that this cytoplasmic enzyme may not have the opportunity to metabolize the norepinephrine that is synthesized by DBH within secretory granules.

Sympathetic neurons are known to change the neurotransmitters they make in response to certain environmental cues. For example, the neurons that innervate the sweat glands in the toe pads of rodents initially use catecholamines as their neurotransmit-

ter, but shortly after their nerve terminals reach the sweat glands, they switch and begin to synthesize acetylcholine instead. This type of switching can be mimicked in culture by a variety of molecules, including cholinergic differentiation factor (CDF, also known as leukocyte inhibitory factor) and ciliary neurotrophic factor. Thus, in one experiment, the DBH promoter was used to direct CDF expression to all sympathetic neurons. However, all the transgenic mice died at birth, perhaps indicating that massive neuronal switching is not compatible with maintenance of vital functions.

In the next study, using the insulin gene promoter, CDF expression was directed to pancreatic islets, a normal target of sympathetic neurons, to ascertain whether these neurons would switch transmitters *in vivo*. In most experiments the mice were crossed with others expressing NGF from the insulin promoter. NGF expression results in massive sympathetic innervation of the pancreas, thereby facilitating the analysis.

Measurement of tyrosine hydroxylase, the rate-limiting enzyme in catecholamine biosynthesis, and of catecholamine levels in pancreas indicated that the neuronal content of both was greatly decreased. Furthermore, choline acetyltransferase activity, the rate-limiting enzyme in acetylcholine biosynthesis, was greatly increased, indicating that switching had occurred. These experiments are being pursued to determine when switching occurs and whether other molecules will function similarly.

Metallothionein Gene Regulation

The most important observation in the area of metallothionein (MT) gene regulation is the discovery of a new member of the MT gene family. A Japanese group recently discovered a small protein that they called growth inhibitory factor (GIF), which is deficient in people with Alzheimer's disease. They showed that brain extracts from these people will support neuronal survival in culture better than control extracts and that GIF inhibits this activity. They purified the protein and published its amino acid sequence.

GIF is remarkably similar to MT, except that it is seven amino acids larger, having two insertions. The human and mouse genes encoding this protein were cloned and shown to resemble the other known mammalian MT genes in intron/exon structure. Furthermore, these genes, now called MT-III, are closely linked to the other MT genes on human chromosome 16 and mouse chromosome 8. The genes appear to be expressed exclusively in glial cells (astrocytes) in the brain. Transgenic mouse ex-

periments are under way in which MT-III expression will either be increased or eliminated, to help define the function of this gene and its potential role in neural degenerative diseases such as Alzheimer's.

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GENETIC AND MOLECULAR DISSECTION OF SIGNAL TRANSDUCTION PATHWAYS

NORBERT PERRIMON, PH.D., *Assistant Investigator*

Although biochemical analyses of signal transduction molecules have been important in demonstrating functional capabilities, they have often failed to reveal how these molecules actually work *in vivo*. Many signal transduction components function promiscuously in *in vitro* assays, interacting with a broad spectrum of substrates. Applying the tools of genetics to establish the epistasis between proteins involved in signal transduction will establish the *in vivo* relationships between signal transducers. Dr. Perrimon and his colleagues have undertaken a molecular genetic analysis of two signal transduction pathways implicated in cell fate determination in the *Drosophila* embryo. The long-term goal is to identify the components involved in these pathways and test their interactions, in order to decipher how a morphogenetic signal controls gene expression in the receiving cells.

The Terminal Signal Transduction Pathway

The establishment of cell fates along the anterior-posterior axis of the *Drosophila* embryo is under the control of three groups or systems of maternally expressed genes: the anterior, posterior, and termi-

nal systems. Genetic analysis of the terminal system supports a model whereby localized activation of the *torso* receptor tyrosine kinase at the egg poles triggers a phosphorylation cascade that ultimately controls the expression of the *tailless* and *huckebein* transcription factors. Dr. Perrimon and his colleagues have characterized two signal transducers of *torso*: *D-raf*, the *Drosophila* homologue of the mammalian serine/threonine kinase *Raf-1*, and *corkscrew* (*csw*), which encodes a novel nonreceptor protein-tyrosine phosphatase containing two SH2 domains. Genetic experiments and studies of downstream gene expression in mutant embryos support a model in which *D-raf* is the signal transducer of the membrane-bound receptor tyrosine kinase encoded by *torso*, while *csw* acts by upregulating the activity of *D-raf*. In addition, Dr. Perrimon and his colleagues have recently shown that *D-ras 1* operates upstream of *D-raf* to transduce the *torso* signal.

Using *in vivo* functional assays, Dr. Perrimon and his colleagues have demonstrated that *D-raf* is the true *Drosophila* homologue of mammalian *Raf-1*. Experiments are in progress to test whether the *csw*

protein is the *Drosophila* homologue of the mammalian *SHPTP2* protein, with which it shares 62% overall identity.

To elucidate the function of the *D-raf* and *csw* proteins, Dr. Perrimon's laboratory has undertaken a structure-function analysis of these proteins. The *D-raf* protein contains three highly conserved domains: a cysteine-rich region, speculated to play a role in protein-protein interactions; a serine/threonine-rich region of unknown function; and the catalytic kinase domain. To gain insights into the functions of each of these domains, the laboratory will introduce a number of modifications into the *D-raf* protein. These engineered mutants will be assayed *in vivo* for their ability to rescue all or parts of the *D-raf* mutant phenotypes. Similarly, a number of *csw* derivatives have been constructed and are currently being assayed for their ability to rescue the *csw* mutant phenotypes. These include modifications in the two SH2 domains and the tyrosine phosphatase catalytic region.

A number of approaches are being used to identify and characterize additional components of this signal transduction pathway. First, using mutations with residual activities in *D-raf* and *csw*, Dr. Perrimon and his colleagues have identified genetically a number of second-site interacting loci. Future work on these genes should identify either novel proteins or previously known proteins that function in *torso* signaling. Second, using the "two-hybrid" technique developed in the laboratories of Drs. Stanley Fields and Roger Brent, which allows the cloning of unknown proteins that associate with a known protein, Dr. Perrimon and his colleagues will attempt to isolate proteins that physically interact with *D-raf*.

The *wingless* Segment Polarity Pathway

Intrasegmental patterning in the *Drosophila* embryo is regulated by cell-cell communication. One signaling pathway that specifies positional information throughout the segment is mediated by the secreted *wingless* (*wg*) protein, which shows significant homology to the mammalian *Wnt-1* protein. The early role of *wg* is to stabilize the expression of the *engrailed* (*en*) homeodomain protein by initiating a phase of *en* autoregulation in the adjacent cells. Dr. Perrimon and his colleagues are studying the role of three genes: *dishevelled* (*dsh*), *porcupine* (*porc*), and *zeste-white-3* (*zw3*) in *wg* signaling. Their working hypothesis is that *porc* is required for the secretion of the *wg* protein and *dsh* and *zw3* are involved in transducing the *wg* signal.

Dr. Perrimon and his colleagues have shown that *zw3* acts as a repressor of *en* autoregulation, and genetic epistasis experiments indicate that *wg* sig-

naling operates by inactivating the *zw3* repression of *en* autoactivation. At least three different serine/threonine protein kinases that diverge at their amino termini are encoded by *zw3*. The differences among the three proteins most likely involve the regulation of *zw3* proteins rather than their target specificities, since constitutive expression throughout development of a single *zw3* protein is able to rescue all aspects of the *zw3* mutant phenotype. *zw3* is 85% identical to the mammalian protein glycogen synthase kinase-3 (GSK3), a known repressor of transcription factors. Dr. Perrimon and his colleagues have demonstrated that *zw3* encodes the *Drosophila* homologue of mammalian serine/threonine kinase GSK3. (A grant from the March of Dimes provided support for the project described above.)

Genetic interactions between the segment polarity genes indicate that the effect of *wg* on *zw3* is mediated by *dsh*, which encodes a novel protein whose structure and sequence have failed to reveal any clues about its function. Using the *Drosophila dsh* gene as a probe, the laboratory, in collaboration with Dr. Daniel Sussman (Lake Placid, New York), has isolated vertebrate homologues, suggesting that *dsh* function has been conserved during evolution. (A grant from the National Institutes of Health provided support for the project described above.)

The *dsh*, *porc*, and *zw3* segment polarity loci were identified as the outcome of screens designed to analyze the maternal-effect phenotypes of zygotic lethal mutations. These screens were made possible by the use of the dominant female sterile (DFS) technique that so far has remained limited to the X chromosome (one-fifth of the *Drosophila* genome). To isolate novel autosomal segment polarity loci, Dr. Perrimon and his colleagues have recently extended the original DFS technique to the autosomes. In addition, the efficiency of the original technique has been improved using the site-specific recombination system developed by Dr. Kent Golic and Dr. Susan Lindquist (HHMI, University of Chicago).

Many genes have been identified that when mutated generate a segment polarity phenotype. Their gene products range from transcription factors to structural proteins, kinases, transmembrane proteins, and secreted factors. In most cases the relationships between the segment polarity genes have been difficult to analyze, because more than one signaling pathway likely operates during segmentation. In addition, analyses of the epistatic relationships between the segment polarity genes have been difficult because of the lack of dominant mutations. To generate embryonic dominant phenotypes associated with the segment polarity genes, Dr. Perrimon and his colleagues will use the Gal4 system of

gene activation. In these experiments they will alter the expression levels or domains of expression of segment polarity genes that have been speculated to act as signaling molecules (*wingless*, *hedgehog*), receptors (*patch*), and transcription factors (*Cubitus*[interruptus], *gooseberry*). These experiments will allow them to test the epistatic relationship between the various segment polarity genes.

Dr. Perrimon is also Assistant Professor of Genetics at Harvard Medical School.

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MOLECULAR GENETICS AND CHROMOSOME STRUCTURE

Stephen T. Reeders, M.D., *Associate Investigator*

The molecular genetics of inherited renal disease is the major focus of Dr. Reeders' laboratory.

Autosomal Dominant Polycystic Kidney Disease (ADPKD)

One of the most common human genetic diseases, ADPKD affects at least 1 in 1,000 adults. Current evidence suggests that hyperplasia of the renal tubular epithelium leads to progressive dilatation of tubules and irreversible deterioration in renal function; dialysis or transplantation are usually required. In addition, recent work suggests that in this disease several proteins normally found on the basolateral surface of the polarized epithelium are found on the apical surface in renal cysts.

To understand the underlying genetic mechanism of ADPKD, Dr. Reeders and his colleagues are attempting to isolate *PKD1*, the common locus mutated in the disease. In addition, they have shown that a second locus, *PKD2*, is occasionally mutated, but that these mutations produce a milder form of the disease. Isolation of these genes is a prerequisite for the study of their protein products and of the way in which mutations disturb the functions of these products.

The genomic region containing the *PKD1* gene lies in a 450-kb segment of chromosome 16, band p13.3. This segment has been cloned into a series of overlapping cosmids. Twenty-three cDNAs have been isolated from the region. Most of these genes are housekeeping genes that are expressed in the kidney and most other tissues. Sequence analysis has shown that all of these genes are novel. They include a gene encoding a zinc finger protein, a cyclin gene, a *ras*-like gene, and a gene whose product has similarities with β subunits of G-binding proteins. The laboratory is currently using single-strand conformation analysis to screen these genes for disease-associated mutations and differences in chemical cleavage specificities.

Dr. Reeders has also occasionally observed onset of ADPKD in neonates and young children. This suggests that the disease may be caused by an unstable repeat element similar to the ones found to cause the fragile X syndrome, myotonic dystrophy, and Kennedy syndrome. Several trimeric repeats have been identified from the region, and these are under investigation. (A grant from the National Institute of Diabetes and Digestive and Kidney Diseases supported the work described above.)

Hereditary Nephritis

A defect in glomerular basement membrane (GBM), one of the major components of the renal filtration mechanism, is responsible for the progressive deterioration in kidney function in hereditary nephritis (Alport syndrome). The common form of this disease is caused by a mutation in the $\alpha 5$ chain of type IV collagen. Dr. Reeders and his colleagues have identified a second form of the disease characterized by autosomal recessive inheritance. In addition, members of the laboratory have cloned two novel type IV collagen chains, $\alpha 3(IV)$ and $\alpha 4(IV)$, which are frequently absent from the basement membranes of patients with Alport syndrome. These genes, which have been mapped to human chromosome 2, are linked to the recessive form of the disease, suggesting that mutation of one or both of these genes in a homozygous state also causes hereditary nephritis. Dr. Mariko Mariyama has shown that the $\alpha 3(IV)$ and $\alpha 4(IV)$ chains are arranged in a head-to-head fashion and has isolated the genes on a yeast artificial chromosome so that further characterization of the mutations can be expedited.

The $\alpha 3(IV)$ chain is also the target for autoantibodies in Goodpasture syndrome, a rare disorder in which autoimmune destruction of glomerular and pulmonary basement membranes is observed. In collaboration with Dr. Billy Hudson (University of Kansas), the B cell epitope has been localized within the noncollagenous domain of the $\alpha 3(IV)$ chain. Further work on the expression of the chain and derivative mutants will permit characterization of the immune reaction in this disease.

Dr. Reeders is also Associate Professor of Internal Medicine and of Genetics at Yale University School of Medicine.

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MOLECULAR GENETICS OF RNA PROCESSING AND CIRCADIAN RHYTHMS

Michael Rosbash, Ph.D., *Investigator*

Dr. Rosbash's laboratory has been engaged for some time in two different areas of investigation. The first is RNA processing and the second is circadian rhythms. The common thread is the approach, which emphasizes conventional and molecular genetics.

RNA Processing

The two post-transcriptional events of interest to Dr. Rosbash and his colleagues are pre-mRNA splicing and transport of mRNA from the nucleus to the cytoplasm. There is evidence to suggest that these two processes are competing—i.e., assembly of a pre-mRNA transcript into a splicing complex prevents RNA transport to the cytoplasm until splicing is successful. As a consequence, both nuclear retention as well as RNA transport must be studied to understand the flow of information from nucleus to cytoplasm.

There is considerable evidence that the REV gene product of the human immunodeficiency virus (HIV) promotes transport of unspliced, or partially spliced, viral RNA to the cytoplasm. It has proved difficult to ascertain whether REV functions by actively promoting RNA transport or by inhibiting the nuclear retention normally afforded by the splicing machinery. To distinguish between these two possibilities, Dr. Rosbash and his colleagues are attempting to recapitulate the REV system in yeast so that genetic methods can be used to identify the proteins with which it interacts. They are also working with several yeast mutants that appear to perturb the normal transport of mRNA from nucleus to cytoplasm.

Of most interest at present is the gene *PRP*20. Its characteristics and its mammalian relatives suggest a connection between transcription on the one hand and RNA transport on the other. This connection between splicing and RNA processing is reinforced by biochemical approaches, which indicate that pre-mRNA splicing in yeast takes place on nascent transcripts. This has significant implications for cell biological experiments that localize splicing components to subnuclear domains in both yeast and mammalian cells.

A second RNA-processing project is focused on the more biological events of pre-mRNA splicing—namely, those associated with splice site recognition. A major problem in splicing is how splice sites are recognized and how splice site partners are assigned, e.g., which 5' site will be attached to which 3' site. *In vivo* experiments indicate that nonconserved sequences within the pre-mRNA are important for splice site partner assignment. Experiments in progress are designed to define the biochemical mechanism responsible for this role of nonconserved sequences.

It is also known that U1 snRNP (small nuclear ribonucleoprotein) is very important in these early events of intron recognition. It is the first splicing component to interact with the pre-mRNA during *in vitro* assembly of the spliceosome, the large complex within which the cleavage and ligation events of splicing take place. The Rosbash laboratory is currently engaged in both biochemical and genetic experiments designed to further understanding of the role of U1 snRNP and its interaction with the pre-mRNA substrate. These experiments are also beginning to identify the protein components of yeast U1 snRNP, most of which remain undefined. (This project was supported by a grant from the National Institutes of Health.)

Circadian Rhythms

The other major interest of Dr. Rosbash and his colleagues is circadian rhythms. To understand the biochemical mechanisms that underlie biological

rhythms, both biochemical and molecular genetic approaches are being used to study mutants of *Drosophila melanogaster* that manifest aberrant or defective rhythms. A major emphasis continues to be on analyzing the function of the period gene (*per*) and the role it plays in the generation or maintenance of circadian rhythms. Mutations in *per* give rise to flies that have fast rhythms, slow rhythms, or essentially no rhythms. Recent work from the Rosbash laboratory suggests that the gene product functions to modulate transcription and that this function is important for the running of the circadian clock.

The *per* product is a nuclear protein and has homology with several known or suspected transcription factors from mammals and from *Drosophila*. It also negatively modulates its own transcription, a feedback loop that is likely to play an important role in circadian clock function. *In vitro* and *in vivo* experiments indicate that the *per* protein undergoes homotypic interactions and suggest that it undergoes heterotypic interactions as well.

The *per* gene product contains no known DNA-binding region, and all indications are that it does not bind DNA. As a consequence, the current working hypothesis is that these putative heterotypic interactions serve to inhibit one or more *bona fide* transcription factors, including one that acts on its own gene. This would generate the negative feedback loop described above and would also affect clock output functions important for the manifestation of rhythm phenotypes.

Current work is designed to identify the partners with which the *per* protein undergoes these proposed heterotypic interactions. These and other experiments should deepen understanding of the gene's function as well as the regulatory loops important for this ubiquitous timing system.

The second rhythm project is designed to uncover new *Drosophila* genes that affect circadian phenotypes. Currently under investigation are several genes that were uncovered in a recent genetic screen for novel mutants that affect circadian rhythms. These are being characterized for their effects on central oscillator function as well as on the phenotypic outputs commonly measured as circadian rhythm indicators. The two genes of most interest at present are likely to act downstream of the central circadian pacemaker. Although the mutant flies are behaviorally arrhythmic, the circadian oscillations of *per*'s mRNA levels occur normally. The laboratory continues to pursue genetic screens to identify additional rhythm-related genes. (This project was supported by a grant from the National Institutes of Health.)

Dr. Rosbash is also Professor of Biology at Brandeis University, Waltham, and Adjunct Professor of Molecular Biology at Massachusetts General Hospital, Boston.

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MOLECULAR ANALYSIS OF DEVELOPMENT IN *DROSOPHILA MELANOGASTER*

SHIGERU SAKONJU, PH.D., *Assistant Investigator*

Drosophila melanogaster is a metameric organism showing unique segmental characteristics. These segmental identities are determined by regulatory functions of homeotic genes during development. Research in Dr. Sakonju's laboratory is focused on understanding, at the molecular level, how these key regulatory genes direct cells to take on appropriate developmental pathways.

Transcriptional Regulation by Homeotic Proteins UBX and ABD-A

Homeotic proteins bind to specific DNA sequences and regulate transcription of target promoters *in vitro* and in cultured cells. However, little is known about mechanisms of transcriptional regulation by homeotic gene products during development. Dr. Sakonju and his colleagues have ana-

lyzed *in vivo* mechanisms of transcriptional regulation by the homeotic proteins UBX (Ultrabithorax) and ABD-A (abdominal-A). The P2 promoter of the *Antennapedia* (*Antp*) gene was a candidate for target, since its expression is repressed by *Ubx* and *abd-A* in abdominal segments. This promoter binds purified UBX and ABD-A proteins at 40 sites, each site showing a core TAAT sequence, within 2.1 kb of the P2 promoter transcription start site. The functional significance of these *in vitro* binding sites was tested by creating point mutations in the core binding sequences, introducing the mutated promoters, fused to a reporter gene, into the fly genome, and examining their expression patterns in developing embryos.

These studies have revealed that UBX and ABD-A proteins use two distinct mechanisms, depending on cell type, to regulate this target promoter. In neuronal cells of the embryonic ventral nervous system, a positive transcription factor, most likely ANTP protein itself, binds to the consensus TAAT sites and activates transcription from the P2 promoter. When UBX and ABD-A are present in these cells, they compete for the binding sites and replace the positive factor, thereby turning off the promoter.

In tracheal cells, an entirely different mechanism is used to repress this promoter. In these cells, UBX and ABD-A bind to multiple sites within the promoter and inhibit a positive transcription factor that acts at a distance through an independent site(s). In the latter mechanism, what matters for repression is the total number of binding sites available for UBX and ABD-A (>15 are required), and not specific locations of the binding sites. This suggests a nonspecific mechanism by which the homeotic proteins can make a promoter inaccessible to different positive transcription factors.

Phenotypic Suppression

When UBX protein is ectopically expressed throughout the embryo via an inducible promoter, head and thoracic segments assume the UBX-specified identity, that of the first abdominal (A1) segment; however, no transformations of abdominal segments posterior to A1 are observed. This resistance of more-posterior segments to transformation by ectopic UBX protein has become known as phenotypic suppression. As an explanation for this phenomenon, others have proposed a functional hierarchy model: when present in the same cells, the homeotic proteins ABD-A and ABD-B, normally expressed in posterior abdominal segments, override the effect of ectopic UBX protein. Dr. Sakonju and his colleagues have tested this model by ectopically expressing ABD-B and UBX proteins, both individu-

ally and simultaneously, throughout the embryo. Contrary to the expectations of the hierarchical model, the ABD-B–induced phenotype (the formation of tail structures called filzkörper) does not prevail over the UBX-induced phenotype. Rather, predominance of each phenotype depends on the relative number of copies for inducible *Ubx* and *Abd-B* genes. Therefore, competition—rather than a functional hierarchy—explains phenotypic suppression.

To explain why ectopic UBX competes poorly in abdominal segments, Dr. Sakonju and his colleagues have proposed and tested a model in which the identities of the abdominal segments become refractory to transformation by ectopic UBX because they are determined earlier in development than those of the thoracic segments. In the test, UBX protein was ectopically expressed at different times during embryogenesis. When it is expressed very early in development, abdominal segments become transformed partially by ectopic UBX. However, ectopic expression at later times does not affect the posterior segments. These results support their model that the identities of more-posterior segments are determined earlier than those of thoracic segments. The laboratory is currently testing a hypothesis that the time of determination is related to the length of transcription units for various homeotic genes.

Isolation of Candidate Genes
That Are Targets of Homeotic Proteins

Homeotic genes specify identities of body segments by regulating expression of other genes. To identify genes that function downstream of homeotic genes, Dr. Sakonju and his colleagues have isolated a number of genes that respond to homeotic gene action. Two methods have been used to isolate such genes.

In the first method, a high degree of DNA sequence conservation allowed isolation of two members of a class of transcription factors called POU domain genes. In other organisms, POU domain genes have been shown to participate in determining identities of specific cell types. The expression patterns of the two *Drosophila* POU genes suggest that they respond to positional cues provided by homeotic genes. RNA *in situ* analyses show that they are expressed in a segment-specific fashion in the embryonic ventral nervous system: at high levels in thoracic segments but low levels in abdominal segments. In the peripheral nervous system, they are also expressed in segment-specific patterns in neurons and their support cells. In mutant embryos that lack the activities of homeotic genes *Ubx*, *abd-*

A, and *Abd-B*, the POU domain genes become derepressed in the abdominal segments of the ventral nervous system. Similarly, the expression patterns in the peripheral nervous system of the abdominal segments transform to that of thoracic segments. Therefore these POU domain genes behave as downstream genes of the homeotic gene functions. An immediate goal is to determine if this regulation is carried out directly by the homeotic proteins.

The second method used to isolate candidate downstream genes was the enhancer-trap screen. In this method a transposon carrying a promoter and a reporter gene is inserted randomly around the genome to identify cis-regulatory elements that respond to homeotic gene activities. A number of candidate genes from this screen are being analyzed. One insertion, which has been shown to map at a gene called *dachsous*, displays a strong *dachsous* phenotype when in trans over existing *dachsous* alleles. This gene, which has been cloned and sequenced (in Dr. Corey Goodman's laboratory [HHMI, University of California, Berkeley]) encodes a large, transmembrane protein containing cadherin repeats.

Dr. Sakonju is also Assistant Professor of Human Genetics at the University of Utah School of Medicine.

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CARDIAC MYOSIN MUTATIONS AND HYPERTROPHIC CARDIOMYOPATHY

JONATHAN G. SEIDMAN, PH.D., *Investigator*

Familial hypertrophic cardiomyopathy (FHC) is an autosomal dominant disorder characterized by unexplained myocardial hypertrophy. The clinical presentation is quite variable but can include syncope, arrhythmias, congestive heart failure, and sudden death. Diagnosis in young people is particularly important, as the incidence of sudden death appears higher in this group and may be the presenting symptom. Indeed, in many series, hypertrophic cardiomyopathy is one of the most common autopsy findings among young athletes who die suddenly. Most of these were undiagnosed previously. Diagnosis in this age group may be particularly difficult, since the diagnostic clinical and echocardiographic criteria may not be manifest until adulthood.

Because mutations in the cardiac myosin heavy-chain (MHC) genes were implicated as the cause of FHC in two families, these genes were analyzed in affected persons from other families. During the past year Dr. Seidman and his colleagues made considerable efforts to find the cardiac MHC mutations that cause FHC in more than 30 unrelated families.

The laboratory first demonstrated that most FHC mutations are missense or point mutations in the MHC genes. A variety of techniques have been successfully employed in the detection of missense mutations within genes. Most of these are based on amplification of genomic DNA sequences and analyses of individual exons. Application of these approaches to the study of FHC mutations was more difficult because the β-MHC polypeptide is encoded in 40 exons, and hence 40 independent analyses are required to examine the entire gene. Furthermore, FHC is an autosomal dominant disorder and affected individuals are heterozygous, bearing one mutated and one normal gene. Genomic analyses may fail to detect deletions of entire exons or mutations that alter gene splicing because of the presence of one normal gene. Access to messenger RNA (mRNA) in which intronic sequences have been excised would overcome these limitations and allow analysis of coding regions in a more rapid and convenient manner.

Although MHC mRNAs are abundant in the heart, expression elsewhere is low, and restricted to selected fibers in slow-twitch skeletal muscle. Normal and mutant β-MHC sequences were detected in RNA transcripts from peripheral lymphocytes and Epstein-Barr virus–transformed lymphocyte cell lines. This finding permitted examination of β-cardiac MHC mRNA, even though cardiac tissue was not available. (These studies were supported in part by a grant from the National Institutes of Health.)

In the first study, seven different β-cardiac MHC mutations were found among 24 unrelated FHC probands. Four mutations were identified in two or

more families. One of these, the Arg453Cys mutation, probably occurred independently in families B and E, because only family B also contains a hybrid α/β-cardiac MHC gene on the same chromosome. Whether other mutations that are shared between apparently unrelated individuals arose independently within mutational hotspots or represent a founder mutation is uncertain.

Characterization of other families should also elucidate whether there are a restricted number of mutations that can cause the FHC phenotype. The identification of seven different mutations in a disease with significant morbidity and premature death suggests that many of these are new and have appeared relatively recently in human evolution. Since FHC mutations do not provide a selective advantage, but have not been lost in the population, they probably reflect a high incidence of new mutational events in the β-cardiac MHC gene.

This hypothesis was tested by examining the β-MHC mRNA sequences of individuals with sporadic hypertrophic cardiomyopathy for missense mutations. Sporadic hypertrophic cardiomyopathy rather than FHC affects 10–30% of individuals with hypertrophic cardiomyopathy. The sporadic form is clinically indistinguishable from the familial form. However, unlike probands with FHC, one of whose parents is affected with the disease, neither parent of a sporadic hypertrophic cardiomyopathy proband is affected.

Examination of β-MHC sequences of seven individuals with sporadic hypertrophic cardiomyopathy revealed two patients with β-MHC missense mutations. Neither parent of either patient had the mutations in their genomes. Apparently new mutations in their β-MHC genes caused the disease in both probands. This provides the most convincing evidence to date that β-MHC missense mutations can be responsible for hypertrophic cardiomyopathy. Dr. Seidman and his colleagues believe that these observations will have considerable implications for future diagnosis and treatment of patients with the sporadic form of the disease.

The natural history of FHC is quite variable, and diagnostic tests have been unable to predict those with a more serious prognosis—those at risk, for example, of sudden, unexpected death. To determine whether particular β-cardiac MHC gene mutations correlate with clinical outcome, the laboratory compared several indices with genotype. Data from families with the same mutation are pooled. Disease-related deaths were infrequent in families with the Val606Met mutations when compared with families that have the Arg249Gln, Arg403Gln, or Arg453Cys mutations. While the incidence of

disease-related deaths in individuals with the Arg249Gln mutation is similar to that associated with other mutations, the average age at death is significantly older for affected individuals within this family.

To assess cumulative survival of affected individuals with respect to age, Dr. Seidman and his colleagues compared survival curves of FHC families with five different mutations for which sufficient numbers of affected persons (alive or deceased) were available. These analyses confirmed that persons with the Val606Met mutation survive longer than those with the Arg453Cys or Arg403Gln mutations. The Arg249Gln mutation appears to produce an intermediate phenotype. Survival with this mutation is better than with Arg453Cys or Arg403Gln. While survival appears shorter in persons with the Arg249Gln mutation than in those with the Val606Met mutation, the difference is not statistically significant. Individuals with the Arg453Cys mutation (with or without the hybrid gene) and those with the Arg403Gln mutation have similar life expectancies, both dying prematurely.

The value of identifying FHC mutations was further demonstrated by analysis of a large family affected by mutation Arg249Gln. Related adult family members were clinically evaluated for FHC, and blood samples were obtained for independent genetic diagnosis. There was complete concordance between the clinical and genetic diagnoses. Thirteen children (ages 2–20) were also evaluated, and while statistically half should be affected, only one child had clinically demonstrable FHC. Genotype analysis of these children revealed six who inherited the mutant MHC gene. These data underscored the insensitivity of clinical diagnostic criteria for FHC in children and young adults. Genetically based diagnoses of FHC permit preclinical diagnosis and should facilitate prenatal diagnosis. Furthermore, the ability to make a preclinical diagnosis in families makes possible longitudinal studies of disease development and interventional trials.

Dr. Seidman is also Professor of Genetics at Harvard Medical School.

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REGULATION OF GENE ACTIVITY DURING B CELL DEVELOPMENT

HARINDER SINGH, PH.D., *Assistant Investigator*

The B cell developmental pathway represents an excellent model for understanding the molecular basis of differentiation of a defined lineage in mammals. This pathway is characterized by an ordered progression of DNA rearrangements that result in sequential expression of immunoglobulin (Ig) genes. B lymphocytes are produced from pluripotent hemopoietic stem cells in the fetal liver and the adult bone marrow. Progenitor B (pro-B) cells initiate rearrangement of the Ig heavy-chain locus and develop into precursor B (pre-B) cells expressing μ protein.

Pre-B cells have been recently shown to express four additional lineage-restricted genes (VpreB, $\lambda5$, *mb-1*, and B29) that encode Ig-associated proteins. The VpreB and $\lambda5$ products represent surrogate light-chain proteins that associate with the μ protein on the surface of differentiating pre–B cells. The *mb-1* and B29 gene products are integral membrane proteins that are associated with the μ receptor on pre–B cells and the Ig receptor on B cells. Pre–B cells differentiate into mature B cells by productive

rearrangement and expression of one of two light-chain loci, κ or λ. B cells can then be triggered by antigen binding and T cell signals to differentiate terminally into Ig-secreting cells.

Projects in Dr. Singh's laboratory are motivated by the assumption that transcriptional control of the activities of B-lineage–specific genes represents critical regulatory steps in B cell development and function. It follows therefore that transcription factors implicated in regulating the activities of B-lineage–specific genes represent key components of the network that orchestrates B cell differentiation. The experimental "logic" underlying this analysis of the molecular control of B cell development is as follows: 1) identify a lineage-specific gene that is essential for B cell development and/or function, 2) show that expression of this gene is regulated at the level of transcription, 3) identify and characterize cis-acting elements of this gene that regulate its transcription, 4) identify and characterize transcription factors implicated in regulation of transcriptional activity, 5) obtain molecular clones of the

relevant transcription factors, 6) use these clones to perturb expression of the transcription factors (anti-sense/gene-disruption methodologies) in order to analyze their regulatory functions, and 7) analyze signaling pathways that control the expression or activation of these transcription factors during B cell ontogeny.

Dr. Singh's laboratory has been focusing on the regulation of transcription of genes encoding components of the B cell antigen-receptor complex. Specifically, they have explored the role of the Oct-2 protein in controlling the expression of the μ, κ, and B29 genes. They have also undertaken an analysis of the control of *mb-1* gene activity. An elucidation of the nature of the regulatory circuitry underlying the expression of these key B cell–specific genes should provide fundamental insight into B lymphocyte differentiation.

Regulation of Immunoglobulin Gene Transcription

Multiple cell-type–specific enhancer and promoter elements mediate B-lineage–restricted transcription of Ig genes. Immunoglobulin (μ, κ, and λ) and B29 gene promoters contain a highly conserved octanucleotide element (ATTTGCAT) positioned ~70 bp upstream of the transcriptional start site. The octamer element is essential for expression of Ig gene constructs in B cell lines, as well as in transgenic mice, and also confers B cell–specific activity to a heterologous promoter. However, octamer elements are also found in the promoters of ubiquitously expressed genes and function in different regulatory capacities depending on promoter context. The identification of the octamer binding proteins Oct-1 and Oct-2 has suggested an explanation for how the octamer motif plays a central role in both lymphoid-restricted and ubiquitous gene expression. The Oct-1 protein was detected in a variety of cell types, thereby suggesting its involvement in the regulation of ubiquitously expressed genes. Oct-2 was detected only in B-lymphoid cell lines and was proposed to mediate the B cell–restricted expression of Ig genes.

Oct-1 and Oct-2 cDNAs have been isolated. Comparison of the deduced amino acid sequences of the two proteins has revealed a highly conserved domain (POU domain) involved in DNA recognition. As anticipated from biochemical analysis, the Oct-2 gene is expressed selectively, though not exclusively, in the lymphoid system. Oct-2 expression is also detected in the embryonic and adult central nervous system. Overexpression of either Oct-1 or Oct-2 in HeLa cells has demonstrated that only Oct-2 can activate certain B cell–specific, octamer-

containing promoters. Furthermore, experiments with somatic cell hybrids support the conclusion that Oct-2 and not Oct-1 is required to activate an Ig promoter. In myeloma × fibroblast hybrids, the activity of an integrated Ig κ promoter construct is extinguished, and this correlates with suppression of Oct-2 expression. Transfection of an Oct-2 expression vector into these hybrids reactivates the extinguished reporter construct.

Genetic Analysis of Oct-2 Function Reveals Two Cell-Type–Specific Pathways of Octamer-Dependent Gene Activation

To test genetically the requirement for Oct-2 in the expression of Ig genes, Dr. Singh's laboratory has exploited a gene-targeting strategy. Both copies of the Oct-2 gene in the B cell line WEHI-231 have been successfully disrupted by the sequential use of promoterless *neo*- and *gpt*-based targeting vectors. Disruption results in an ~20-fold reduction in Oct-2 protein levels. The low levels of residual expression may be due to the fact that the promoterless targeting vectors required disruption of the first coding exon. Transcripts initiated upstream of the second exon, which also contains an in-frame AUG, might account for residual expression. Nevertheless, given the dramatic reduction of Oct-2 protein levels, the phenotype of the mutant B cells is unexpected. No significant alteration in the expression of μ, κ, and B29 genes is detected in the mutant cells. This result represents the first genetic demonstration that Oct-2 appears not to be necessary for the maintenance of Ig gene expression. These results suggest the existence of an alternate pathway, involving the ubiquitous related protein Oct-1, in Ig gene regulation.

To determine if the double disruptant cells are defective in activating different octamer-dependent transcription units, Dr. Singh and his colleagues have pursued transient transfection analysis. Simple octamer-containing promoters are unaffected by the double disruption, whereas a promoter containing a multiple array of octamer elements is severely compromised in the mutant cells. These results provide strong genetic evidence for two distinct, cell-type–specific pathways of octamer-dependent gene activation in B lymphocytes.

Regulation of Oct-2 Transcription

In pre–B cell lines, expression of the Oct-2 gene is transcriptionally regulated during lipopolysaccharide-induced differentiation. Dr. Singh's laboratory is analyzing the regulation of Oct-2 gene transcription in B-lineage cells, with the aim of identifying and analyzing other regulatory genes that constitute

a genetic hierarchy controlling B cell development. A major promoter of the Oct-2 gene has been identified and sequenced. This region is being functionally analyzed. Furthermore, since the Oct-2 gene is a large locus (~100 kb), it has been surveyed for lineage-specific DNase I–hypersensitive sites. Such sites may represent enhancer or locus control region elements. Two such sites have been localized to large introns separating upstream coding exons. These regions are being tested for function.

Regulation of *mb-1* Gene Activity

The *mb-1* gene encodes an integral membrane protein that appears to be required for the surface expression and signaling function(s) of the Ig receptor on B lymphocytes. The gene, which is expressed in a lineage-restricted manner, is activated early in B cell ontogeny, continues to be expressed in mature B cells, but is turned off in terminally differentiated plasma cells. Dr. Singh's laboratory has identified the *mb-1* promoter and functionally tested its activity by transient transfections. A 737-bp promoter fragment preferentially stimulates accurately initiated transcription in *mb-1*–expressing B cells. Deletion analysis of the promoter suggests the presence of two functional domains—proximal and distal. Both domains independently activate transcription from a heterologous promoter. The distal domain functions in a cell-type– and stage-

specific manner, activating transcription in B cells but not in T cells or plasma cells. A 25-bp element within this domain is necessary and sufficient for activity. This element is recognized by a novel cell-type– and stage-specific transcription factor, termed BLyF. The binding of BLyF correlates with the ability of the regulatory element to stimulate transcription. Thus BLyF appears to positively regulate transcription of the *mb-1* gene. The results also suggest that the inactivity of the *mb-1* locus in plasma cells is not simply due to the loss of BLyF activity.

Dr. Singh is also Assistant Professor of Molecular Genetics and Cell Biology at the University of Chicago.

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DEVELOPMENTAL GENETICS

PHILIPPE M. SORIANO, PH.D., *Assistant Investigator*

Research in Dr. Soriano's laboratory is focused on early development of the mouse embryo, with particular emphasis on mechanisms underlying cell-cell interactions and signaling. The experimental approach involves the derivation of mouse lines carrying mutations in genes implicated in these processes through targeted disruption or promoter traps in embryonic stem (ES) cells.

Genetic Analysis
of Protein-Tyrosine Kinases

Previous work of this laboratory was focused on the derivation of mouse mutants for the c-*src* proto-oncogene. Although the oncogenic variant of the gene had been well characterized, little was known about the essential functions of the normal cellular counterpart. The *src* protein is a nonreceptor tyrosine kinase highly expressed in the central nervous system (CNS) during embryogenesis and in many

cell types in the adult, including cells of hematopoietic origin such as platelets. Surprisingly, disruption of the *src* gene by homologous recombination in ES cells did not lead to embryonic death, but instead to a highly restricted recessive phenotype in the adult, osteopetrosis. This phenotype is characterized by defective osteoclast function leading to a defect in bone remodeling.

To characterize the mutation further, work done in collaboration with Dr. Greg Mundy (University of Texas, San Antonio) has shown that the inherent defect in *src*⁻ mice is confined to the osteoclasts and is cell autonomous, since the defect can be corrected by fetal liver transplants and is therefore independent of the bone marrow microenvironment. These studies may help to identify a substrate unique to *src*. (The project described above was supported by a grant from the National Institutes of Health.)

The absence of a more severe phenotype in *src*⁻

mice might be due to compensation by other genes, as suggested by several lines of biochemical evidence. To test the hypothesis that other kinases in the same family as *src* may be playing overlapping roles, mice carrying mutations in the genes encoding the *fyn* and *yes* kinases have been derived by gene targeting in ES cells. Both mutant mice fail to exhibit an overt phenotype, again probably because of functional compensation. However, further studies of the *fyn* mutants reveal signaling defects with respect to the T cell receptor in thymocytes, and to a lesser extent in mature T cells. In addition, preliminary evidence suggests that the *fyn*⁻ mice may be impaired in their ability to undergo negative selection in the thymus. These studies provide definitive evidence for an essential role of *fyn* in T cell receptor signal transduction.

Work done in collaboration with Dr. Eric Kandel (HHMI, Columbia University) has shown also that *fyn*⁻ mice exhibit specific defects in long-term potentiation (LTP) in the hippocampus, with accompanying abnormalities in learning and memory. Other collaborative work with the laboratory of Dr. Harold Varmus (University of California, San Francisco) is aimed at understanding the role of tyrosine kinases in macrophages in *src* families. To this end, mice deficient for the *hck* and *fgr* kinases have been produced as well.

Mice deficient in more than one kinase have been generated by intercrossing *src, yes*, and *fyn* mutant mice. Consistent with the hypothesis that these kinases are able to compensate for one another, the expected number of double mutants is not recovered from crosses involving two kinase mutants. In some of these crosses, particularly those involving *src*, over 90% of the double mutants die at birth or during gestation. Crosses involving both *fyn* and *yes* have allowed the recovery of a larger fraction of double homozygotes. However, these animals become sick and die of membranoproliferative glomerulonephritis, usually between the ages of two and four months.

Because *fyn* may be involved in negative selection in the thymus, and *yes* is also expressed by thymocytes and T cells, it remains to be seen if these symptoms are due to an autoimmune disease. In another line of work, interactions between nonreceptor tyrosine kinases and neural cell adhesion molecules are being investigated by intercrossing kinase-mutant mice with mice, produced in the laboratory, that carry a mutation in the neural cell adhesion molecule L1. These studies are in progress.

It has been known for some time that *src* and related kinases are negatively regulated by phosphory-lation at the carboxyl-terminal tyrosine. Previous studies from this laboratory and that of Dr. Joan Brugge (HHMI, University of Pennsylvania) had demonstrated that another kinase was responsible for this activity. A candidate kinase, *csk* (c-*src* kinase), has been shown to be functional on *src* and several other members of the *src* family. To examine the consequences of dephosphorylation of the carboxyl-terminal tyrosine, and therefore simultaneous activation of several, if not all, members of the *src* family, Dr. Soriano's laboratory has produced mice carrying a disruption of the *csk* gene. Preliminary observations indicate that this mutation leads to a recessive lethal phenotype. Because neoplasms have not been detected yet in heterozygous animals, it remains unclear whether *csk* acts as a tumor-suppressor gene.

Promoter Traps in ES Cells

The laboratory has been interested in identifying new genes that play critical roles in early development, based on the phenotypic consequences of their inactivation in transgenic mice. Because only 1 out of 20 transgenic strains on the average exhibits a mutant phenotype, a screen based on "promoter traps" was devised. In this screen a reporter gene is placed downstream of a splice acceptor sequence in a retroviral vector. The reporter gene encodes a fusion protein, β-geo, with both β-galactosidase and neomycin phosphotransferase activities. Following infection of ES cells, administration of G418 selects for insertions downstream of a promoter active in such cells. These ES cells are then used to derive transgenic mice in which the activity of the mutated gene can be traced by β-galactosidase expression.

Fifty transgenic lines so derived have now been generated. These lines exhibit different staining patterns during development, ranging from restricted to specific tissues (e.g., CNS or liver) to ubiquitous. Among 42 strains with β-galactosidase activity, 18 exhibit a recessive lethal phenotype due to promoter trap events, indicating the efficiency of this type of screen.

The first gene identified by this approach is transcriptional enhancer factor 1 (TEF-1), yielding a broadly expressed transcription factor. Analysis of mutant mice with a tagged TEF-1 gene has shown that the provirus insertion leads to a null mutation and that homozygous embryos die at midgestation. Further analysis of this and other mutant strains is under way. (The project described above was supported by a grant from the National Institutes of Health.)

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STRUCTURE AND FUNCTION OF SMALL RIBONUCLEOPROTEINS IN MAMMALIAN CELLS

Joan Argetsinger Steitz, Ph.D., *Investigator*

A variety of small ribonucleoproteins (RNPs, tight complexes between one or more proteins and a short RNA molecule) inhabit all higher eukaryotic cells. Many are highly abundant ($>10^5$ copies/cell) and highly conserved across species. Different types are localized specifically in the cell nucleus, nucleolus, or cytoplasm and are often the targets of autoantibodies found in the sera of patients with rheumatic disease. Dr. Steitz's laboratory would like to evolve a full description of both the variety of basic cellular processes that require input from small RNPs and the various ways in which the RNA moieties contribute to the action of individual particles. So far, all small RNPs appear to participate in gene expression or genome maintenance.

Small RNPs Involved in Splicing

The most abundant ($\sim 10^6$/cell) of all small nuclear RNPs (snRNPs) contain U1, U2, U5, or U4 + U6 RNAs and ~6–12 proteins, some unique and some common. These snRNPs belong to the Sm class and are precipitable by anti-Sm patient antibodies. They assemble on the pre-mRNA, together with many additional protein factors, to form a large body called the spliceosome, which carries out the two-step excision of introns from the pre-mRNAs of eukaryotic cells.

New snRNA · snRNA and snRNA · pre-mRNA contacts in the spliceosome. Although mechanistic similarities between group II self-splicing and nuclear pre-mRNA splicing have been apparent for some time, only a few parallels in RNA · RNA interactions between the two systems are known. Two different crosslinking strategies have been used to characterize specific contacts between snRNAs, snRNAs and the pre-mRNA, and proteins and the pre-mRNA during the course of splicing in HeLa cell extracts. In addition to previously suspected or documented interactions, novel contacts between the pre-mRNA

and the U5 and U6 snRNPs have been mapped, and kinetic analyses that allow these interactions to be sequentially ordered have been performed.

1. *Site-specific crosslinking of mammalian U5 snRNP to the 5′ splice site prior to the first step of pre-mRNA splicing.* The standard adenovirus substrate and a spliced leader (SL) RNA-containing substrate were synthesized with a single ^{32}P-labeled photoactivatable 4-thiouridine residue two nucleotides upstream of the 5′ splice site. Selective photoactivation of the 4-thiouridine after incubation of either substrate under splicing conditions in HeLa nuclear extract resulted in crosslinks to the U5 snRNA and the U5 snRNP protein p220. These ATP-dependent interactions occur prior to the first step of splicing. The U5 snRNA crosslinks map to a phylogenetically invariant nine-nucleotide loop sequence but do not require Watson-Crick complementarity to the 5′ exon. The results therefore provide biochemical evidence for a U5 snRNP–5′ exon interaction, previously suggested based on genetic studies in yeast. In addition, crosslinks of this position in the 5′ exon to U1—but not to U2, U4, or U6 snRNAs—are observed and are now being characterized.

2. *Psoralen crosslinking detects specific interactions of U1, U2, U5, and U6 snRNAs with pre-mRNA during* in vitro *splicing.* The crosslinking reagent psoralen has been used to analyze the interactions of snRNAs with an adenovirus pre-mRNA in HeLa cell nuclear extract. An endogenous U2-U4-U6 crosslinkable complex dissociated upon incubation with the splicing substrate. During splicing, U1, U2, U5, and U6 became crosslinked to pre-mRNA, and U2, U5, and U6 became crosslinked to the excised lariat intron. U2 also formed a doubly crosslinked complex with U6 and pre-mRNA. The U1, U5, and U6 crosslinks mapped to intron sequences near the 5′ splice site, whereas the U2

crosslink mapped to the branch site. The kinetics of crosslink formation and disappearance delineates a temporal pathway for the action of small RNAs in the spliceosome. Potential base-pairing between conserved sequences in both the snRNAs and pre-mRNA at the sites of crosslinking argue that the 5′ splice site is inspected by the U5 and U6 as well as the U1 snRNP prior to the first cleavage event. The data further suggest functional analogies between U6 snRNA and domain 5 of group II self-splicing introns.

Multiple functions for the U1 snRNP? There are many indications that the U1 snRNP, which is slightly more abundant than the other splicing snRNPs, may play several roles in splicing as well as in related RNA-processing events.

1. *Two functions for the 5′ end of U1 in pre-mRNA splicing.* To probe functions of the U1 snRNP during *in vitro* splicing, Dr. Steitz and her colleagues examined unusual splicing substrates that replace the 5′ splice site region of an adenovirus substrate with SL RNA sequences from *Leptomonas collosoma* or *Caenorhabditis elegans*. Such chimeric transcripts were previously shown not to require 5′ splice site/U1 snRNA base-pairing and thus uncouple this function from other roles the U1 snRNP may perform in splicing. When antisense 2′-O-methyl oligoribonucleotides were used to sequester stably positions 1–10 of U1 snRNA involved in 5′ splice site recognition, splicing of the SL constructs continued; however, longer oligoribonucleotides that also disrupt the first stem of U1 snRNA inhibit the splicing of the SL as well as the adenovirus substrates. Native gel analysis indicated that the longest oligoribonucleotide inhibits splicing by blocking spliceosome formation subsequent to U2 snRNP association but before joining of the U4/U5/U6 tri-snRNP. Thus, although the extreme 5′ end of U1 snRNA base-pairs with the 5′ splice site, the sequence or structural integrity of stem I is essential for some later function in spliceosome assembly.

2. *U1 snRNP association with terminal exons.* Psoralen crosslinking experiments in HeLa cell nuclear extracts have revealed the binding of U1 snRNA to substrates containing the simian virus 40 (SV40) late or adenovirus L3 polyadenylation signals. The sites of U1 crosslinking to the substrates mapped different distances upstream of the AAUAAA sequence to regions with limited complementarity to the 5′ end of U1 snRNA. U1 crosslinking to the same site in the SV40 late pre-mRNA was enhanced by addition of an upstream 3′ splice site, which also enhances polyadenylation. Examination of different nuclear extracts revealed a correlation between U1 crosslinking and the coupling of splicing and poly-

adenylation, suggesting that the U1 snRNP participates in the coordination of these two RNA-processing events. The mechanism of communication between the splicing and polyadenylation machineries, as well as how interaction of the U1 snRNP with 3′-terminal exons might contribute to mRNA export, is under further study.

Minor snRNPs Related to Splicing snRNPs

In addition to the splicing snRNPs, mammalian cells contain a number of less abundant snRNPs (10^3–10^4 copies/cell), which also have 5′-trimethylguanosine caps on their RNAs and contain proteins reactive with anti-Sm autoantibodies. U11 and U12 are two low-abundance snRNPs that coexist in a two-snRNP complex. Their functions are under study in part using serum from a scleroderma patient that contains novel antibodies that recognize protein component(s) of the U11 particle as well as antibodies against the trimethylguanosine cap of U RNAs. Psoralen crosslinking and genetic suppression approaches are being used to analyze the low-abundance U7 snRNP, which participates in the 3′-end maturation of histone pre-mRNAs.

Mammalian Nucleolar snRNPs

Mammalian cell nucleoli contain a family of related small RNP particles, whose RNAs are called U3, U8, U13, U14, U15, and U16. Human U8 and U13 are present at about $^1/_5$ and $^1/_{20}$ the amount of U3 (10^6/cell), respectively, and, like U3, contain 5′-trimethylguanosine cap structures. All these particles can be immunoprecipitated by autoantibodies to fibrillarin, an abundant protein of the fibrillar subcompartment of the nucleolus. To date, only a function for U3 in the earliest processing step of preribosomal RNA maturation to yield 45S pre-rRNA has been established, but the other nucleolar snRNPs are likewise believed to participate in ribosome biogenesis.

Nucleolar targeting of snRNPs. The requirements for import of the human U3 snRNA into the nucleus have been analyzed in the *Xenopus* oocyte. Neither cap trimethylation nor binding of the common protein fibrillarin were found to be essential. Rather, a structural element that includes the 3′ end of the RNA was identified as a novel determinant for nuclear import. Currently the protein composition of the U3 snRNP is being scrutinized, with the goal of finding proteins (and their binding sites on U3) that are required for nuclear and eventual nucleolar localization of this snRNP.

Finding a function for U8. U8 RNA from *Xenopus* has been sequenced to identify regions conserved with mammalian U8. Some of the highly ho-

mologous stretches of nucleotides reside in regions that can be successfully targeted by RNase H for destruction of the U8 snRNP particle. Injection of complementary deoxyribonucleotides into the *Xenopus* oocyte is therefore being used to ask whether the U8 snRNP (like U3) contributes to pre-rRNA processing. Hints that U8 is involved in a step affecting the appearance of 28S rRNA have recently been obtained.

A gene for U15 resides within an intron of the ribosomal protein S3 gene. Two variants of U15 RNA (formerly called X), which are not trimethyl-guanosine-capped but bind fibrillarin, have been detected in human cells. Curiously, the gene for one of these variants—instead of being present in multiple copies, as for other U RNA genes—is single-copy. It resides within an intron of the ribosomal protein S3 gene and is encoded on the same strand as the mRNA. Cell-free extracts can process U15 from intron transcripts to yield the same 5′ terminus as native U15, arguing that the nucleolar small RNA and S3 mRNA are both derived from the S3 pre-mRNA. The presence of the gene for a small nucleolar RNA within an intron of a ribosomal protein gene links the production of these two contributors to ribosome biogenesis in mammalian cells.

The work described in the previous three sections is supported by a grant from the National Institute of General Medical Sciences, National Institutes of Health.

Viral snRNPs

Some viruses encode small RNAs that assemble together with host proteins to form snRNPs related to cellular particles. Such RNAs from both *Herpesvirus saimiri* and Epstein-Barr virus are currently under study (supported by a grant from the Cancer Institute, National Institutes of Health). They appear to contribute to cellular transformation, since they are among the relatively few viral gene products expressed in virus-transformed lymphocytes.

Herpesvirus saimiri–encoded U RNAs may stabilize short-lived cellular messages. Marmoset T lymphocytes transformed by *H. saimiri* contain seven novel virus-encoded U RNAs called HSURs (*H. saimiri* U RNAs). HSURs assemble with Sm proteins and acquire a 5′-trimethylguanosine cap, categorizing them as Sm snRNPs, but are of low abundance ($\sim^1/_{50}$–$^1/_{500}$ the level of U1 RNA). Three of the HSURs share 5′-end sequences that exhibit homology to AU-rich sequences found in the 3′-untranslated regions of short-lived mRNAs for certain lymphokines, cytokines, and proto-oncogenes. The possibility that HSURs contribute to cell transformation by inhibiting the selective degra-

dation of (and thereby stabilizing) important cellular messengers is supported by *in vitro* ultraviolet-crosslinking studies, which have revealed that the AU-rich sequence at the 5′ ends of HSURs 1, 2, and 5 binds the same 32-kDa protein as the degradation signals of several oncogene and growth factor mRNAs. Attempts to purify and clone this protein are under way, with the goal of elucidating its function in mRNA degradation.

Epstein-Barr virus–encoded RNA specifically binds ribosomal protein L22. Human B lymphocytes latently infected with Epstein-Barr virus produce large amounts (10^7/cell) of two snRNAs called EBERs (Epstein-Barr–encoded RNAs). The EBERs are transcribed by RNA polymerase III (pol III) and are permanently associated with the La protein, an autoantigen that binds, at least transiently, to all pol III transcripts through an association with their U-rich tails. EBER 1 was recently demonstrated to bind a second highly abundant cellular protein (15 kDa) named EAP (for EBER-associated protein). The cloning and sequencing of a human EAP cDNA revealed that EAP is 77% identical to a previously characterized sea urchin protein that is developmentally expressed but of unknown function. Recently antibodies against EAP were raised and used to determine EAP's binding site on EBER 1 and its subcellular localization. EAP binds to a particular (conserved) stem-loop of EBER 1, recognizing nucleotides in both the single- and double-stranded regions. Immunofluorescent staining of nucleoli and the cytoplasm by anti-EAP antibodies led to the realization that EAP may be a ribosomal protein. Indeed, it has been identified as the large subunit polypeptide L22, in collaboration with Dr. Ira Wool (University of Chicago). Studies are now under way to elucidate why binding a significant fraction ($\sim 50\%$) of a specific ribosomal protein in the infected cell is important to Epstein-Barr virus.

Dr. Steitz is also Henry Ford II Professor of Molecular Biophysics and Biochemistry at Yale University School of Medicine.

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MOLECULAR GENETICS OF NEMATODE DEVELOPMENT AND BEHAVIOR

PAUL W. STERNBERG, PH.D., *Associate Investigator*

Dr. Sternberg's laboratory takes a molecular genetics approach to study basic questions in developmental biology and neurogenetics, using the nematode *Caenorhabditis elegans.* Cell-type specification by intercellular signaling, the genetic control of innate behavior, and the genetic and cellular basis for morphogenesis are among the problems studied. The development and function of the *C. elegans* male copulatory spicules and hermaphrodite vulva provide an opportunity to study these problems. The major strategy is to identify mutations that make cells or animals misbehave and then to study the functions of the genes defined by these mutations, using a combination of molecular biology and genetic analysis.

Male Spicule Development

The concerted morphogenesis of specialized epidermal cells and neural cells forms the two copulatory spicules, innervated structures crucial to male mating with hermaphrodites. Because *C. elegans* hermaphrodites are internally self-fertilizing—each animal producing both sperm and ova—male mating is evidently a dispensable behavior and the spicule a dispensable organ. Each of the two spicules comprises nine cells: two sensory neurons, one motoneuron, and six supporting cells, some of which secrete a hardened cuticle.

A number of mutations that disrupt various aspects of spicule development have been identified. Several mutations are defective in the generation of spicule cells, while others affect the differentiation of the cells and the morphogenesis of the spicules. These mutations are being analyzed to define a genetic pathway specifying spicule development.

By cell ablation experiments, a network of five or more cell interactions crucial to spicule development has been identified. At an intermediate stage of spicule development, eight cells are arranged in two tiers of four cells each. The dorsal tier is induced by a signal from the nearby F and U cells. The ventral tier receives a signal from the Y.p neuroectoblast or its progeny. In addition, there are three or more interactions between the ventral and dorsal tiers and within each tier.

The *lin-3, let-23, let-60,* and *lin-45* genes are necessary for the induction of the dorsal tier by the cells F and U. The *lin-3* gene encodes an epidermal growth factor (EGF)-like growth factor precursor. The *let-23* gene encodes a tyrosine kinase of the EGF-receptor subfamily. The *let-60* gene encodes a *ras* protein. The *lin-45* gene encodes a *raf* serine-threonine protein kinase. Specification of these neuroectoblasts thus involves a highly conserved signal transduction mechanism. By screening for mutants with defective spicules, Dr. Sternberg and his colleagues have identified new mutations that affect spicule development.

Mating Behavior

Male mating, the most complex nematode behavior, is being studied by cell ablation experiments and genetic analysis. A series of steps has been iden-

tified that involve sensory input coupled to motor output: 1) attraction to hermaphrodites, 2) recognition of the hermaphrodite, 3) maintaining contact with hermaphrodites, 4) location of the vulva, 5) insertion of spicules, 6) transfer of sperm, and 7) retraction of spicules. The roles of the male-specific neurons and muscle cells in mediating mating behavior are being examined by killing individual cells or classes of cells and observing the consequences for the behavior. For example, continued backward movement during vulval location requires the PVY interneuron. Vulval location is mediated by the HOA and HOB sensory neurons. Insertion of spicules is mediated by the SPD sensory neuron and SPC motoneurons of the spicule. The SPV spicule sensory neurons are required to prevent sperm transfer until after spicule insertion.

Mutant strains defective in male mating have been isolated. Some of these mutant males have obvious defects in the development of the spicules. Other mutants, called "Cod" (for copulation defective), are anatomically normal yet defective in mating behavior. By studying Cod mutants, the genetic control of each step in this complex behavior can be elucidated.

Most of the initial Cod mutants analyzed are defective at only a single step in the mating process. For example, a mutant male defective in step 5 will locate the vulva, but fail to insert his spicules. This defect is similar to that of males whose spicule neurons have been ablated. Having mutants blocked at defined steps will allow the definition of genes necessary to specify this innate behavior. Based on mapping and complementation studies, most Cod mutations of this first set define separate genes.

The expression of four genes that encode α subunits of heterotrimeric G proteins have been examined. Two of these are expressed in cells required for male mating: *gpa-1*, in either the SPD or SPV spicule sensory neurons; and *goa-1*, which encodes a nematode homologue of mammalian $G_o\alpha$, in the male diagonal muscles as well as a number of male-specific neurons.

Cell-Type-Specific Functions During Vulval Development

The anchor cell induces two distinct types of vulval precursor cells (VPCs). The 1° VPC generates progeny that attach to the anchor cell during vulval morphogenesis. The 2° VPC generates progeny cells in a fixed anterior-posterior orientation. Genes specific to the 1° or 2° VPC lineage are being studied by genetic and molecular techniques. The *vex-1* gene is involved in the divisions of the 1° but not the 2° VPC lineage and is a candidate 1°-specific gene.

The *lin-18* gene is necessary for the orientation and asymmetry of the 2° VPC and is a candidate 2°-specific gene. The "ground state" of a 2° VPC is to orient posteriorly, but in the presence of a signal from the developing gonad it reorients anteriorly. This signal is distinct from the inductive signal from the anchor cell, since mutations defective in inductive signaling do not affect the correct orientation of the 2° VPCs.

Vulval Induction

An inductive signal from the anchor cell stimulates vulval differentiation from nonspecialized epidermis. This inductive signal is encoded, at least in part, by the *lin-3* gene, and is an EGF-like growth factor. The gene is expressed in the anchor cell at the time of vulval induction. The candidate receptor for *lin-3* is the EGF receptor homologue encoded by the *let-23* gene. A number of *let-23* mutant alleles have been sequenced. Two that cause tissue-specific defects in *let-23* function truncate the carboxyl terminus of the protein. The inductive signal is transduced by a number of genes including *let-60 ras* and *lin-45 raf*. Several negative regulators of this pathway have been identified, including a clathrin adaptor protein that is the product of the *unc-101* gene. (This project was supported by a grant from the National Institutes of Health.)

A negative signal, possibly from nearby epidermis, prevents vulval differentiation in the absence of the inductive signal. This signal was defined by mutations in the *lin-15* locus, which result in vulval differentiation in the absence of an inductive signal. The *lin-15* locus has been cloned and found to encode two products required to prevent inappropriate vulval differentiation. (This project was supported by a grant from the March of Dimes Birth Defects Foundation.)

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TRANSCRIPTION FACTORS IN CELL GROWTH AND KIDNEY DIFFERENTIATION

Vikas P. Sukhatme, M.D., Ph.D., *Assistant Investigator*

The focus of Dr. Sukhatme's laboratory is on cloning mammalian transcription factor genes that function in two different contexts. The first relates to regulatory genes whose induction is modulated during the transition of quiescent cells into the G_1 phase of the cell cycle. These immediate-early transcription factor genes are also induced by diverse extracellular signals. A second set of studies is aimed at characterizing transcription factor cascades of import during kidney differentiation. These two projects have recently intersected in studies with the Wilms' tumor gene *WT1*.

EGR Family of Immediate-Early Transcription Factors

Extracellular signals such as neurotransmitters, growth factors, hormones, and matrix are known to be key modulators of cellular phenotype. These agents lead to the generation of second messenger signals in the plasma membrane and cytosol. In turn, these biochemical events modulate the expression of a set of so-called immediate-early genes (IEGs), whose induction does not require *de novo* protein synthesis. Several years ago, Dr. Sukhatme's laboratory and others identified several IEGs primarily in the context of a mitogenic response and more specifically in the transition of a cell out of a quiescent state (G_0) into G_1. Of particular interest to Dr. Sukhatme's laboratory has been a subset of IEGs that encode transcription factors, since as such they might 1) be the targets for second messenger events and 2) activate or repress the transcription of critical genes required to effect a particular cellular phenotype.

In 1987 Dr. Sukhatme's laboratory discovered (concurrently with several other laboratories) the *EGR* family of IEGs. The best-characterized of these genes is *Egr-1* (early growth response gene-1). *Egr-1* (also known as *Zif-268*, *Tis-8*, *NGFI-A*, and *Krox-24*) was isolated as a serum-inducible IEG in quiescent fibroblasts (G_0-G_1 transition), utilizing a differential screening protocol. The gene is induced by mitogenic stimulation in every mammalian cell type tested, including B cells; T cells; kidney mesangial, glomerular, and tubular epithelial cells; hepatocytes; and endothelial cells. The cDNA structure predicts a protein whose carboxyl terminus contains three zinc fingers of the Cys_2-His_2 type, first identified in the *Xenopus* transcription factor TFIIIA.

An exciting part of the *Egr-1* story is that this gene, like c-*fos* and c-*jun*, can be activated in many physiologic contexts in addition to being ubiquitously induced in a mitogenic response. For example, during the past year it was discovered that ionizing radiation can induce *Egr-1* mRNA in certain cell types. *Egr-1* is also an immediate-early gene in this context, and kinase C is critical in this induction. The DNA sequences responsible for this stimulation include the CArG boxes—i.e., the multiple serum response elements present in the *Egr-1* promoter (collaboration with Drs. Donald Kufe [Harvard Medical School] and Ralph Weichselbaum [University of Chicago]). These studies may define radiation-responsive cis sequences that could drive heterologous genes, thus leading to a novel modality for "targeted" gene therapy, with activation controlled exogenously by ionizing radiation.

Dr. Sukhatme and his colleagues have also been interested in structure-function studies of the *Egr-1* protein. Does this protein function as a transcription factor via

binding to its target sequence? A cotransfection assay utilizing an *Egr-1* expression vector and a reporter with three Egr-1–binding sites placed upstream of a minimal promoter and the chloramphenicol acetyltransferase (CAT) gene showed that *Egr-1* is a transcriptional activator. This system is being used to delineate activation domain(s), nuclear localization signals, and DNA-binding requirements. Not surprisingly, the three zinc fingers are sufficient for DNA binding. Nuclear localization depends on a bipartite sequence, one similar to the corresponding signal in the simian virus 40 (SV40) T antigen, the other part of the zinc finger motif. Two activation domains in the amino-terminal region of the protein have been mapped. An unexpected finding is a region of 50 amino acids 5′ of the three fingers, which when deleted from the wild-type protein results in a superactivator mutant. Moreover, this region functions as a modular repressor, as demonstrated by studies with chimeric constructs with the yeast GAL4 (1–147) fragment. This fragment is one of the smallest mammalian repressor "proteins" defined to date. This region, as well as the nuclear localization signals and the DNA-binding domain, is exquisitely conserved in the zebrafish, the earliest evolutionary *Egr-1* homologue found.

Transcription Factors in Early Kidney Development

The molecular events that characterize the development of the kidney are the second focus of Dr. Sukhatme's laboratory. It is well known that metanephric mesenchymal cells convert into epithelial cells over a 4- to 5-day period in response to invasion of the ureteric bud. Little is known at the molecular and cellular level of the events that transpire during this process. Dr. Sukhatme's approach is based on the premise that characterization of a hierarchy of transcriptional regulators that occur during this period will be important in understanding this process. Toward this end, recent studies have focused on the Wilms' tumor gene *WT1*.

The WT1 protein in three of its four zinc fingers shows a 60–70% similarity to the amino acid sequence of the Egr-1 finger domain. Antibodies raised against the Wilms' tumor antibody cross-react with Egr-1, and both proteins can bind to the same target sequence (GCGGGGGCG) (Dr. Frank Rauscher, Wistar Institute). However, WT1 is a negative regulator of transcription, whereas Egr-1 is a positive regulator (in collaboration with Dr. Rauscher).

What then are the physiologic targets for WT1 action in the kidney? Several binding sites exist for the Egr-1/Wilms' tumor proteins in the promoter sequence of insulin-like growth factor II (IGF-II). It

has been known that IGF-II levels are high in Wilms' tumor and that during development IGF-II levels fall. Dr. Sukhatme's laboratory, in collaboration with Dr. Rauscher, used cotransfection studies to investigate the possibility that IGF-II is a direct target for the repressive action of the Wilms' tumor protein, and found this to be the case. Two critical target sites (one upstream and one downstream of the transcription site) were identified by deletion analysis and mutagenesis. Gel shift and footprint analysis provided evidence for a direct interaction, and these sequences were sufficient to confer repression when ligated to a heterologous promoter.

These studies constitute the identification of the first target gene for WT1 and suggest a mechanism for the genesis of some Wilms' tumors. Recently it has been noted that a major alternatively spliced variant of WT1 also represses the IGF-II promoter. This variant, which contains a three–amino acid insertion between the third and fourth zinc fingers, does not bind to the Egr-1 target consensus.

In preliminary studies in collaboration with Dr. Tucker Collins (Harvard Medical School), Dr. Sukhatme and his colleagues have shown that a 900-bp promoter region of the platelet-derived growth factor (PDGF) human A chain is strikingly repressed by WT1. This region of the promoter contains several putative sites for WT1/Egr-1 binding. Studies are currently under way utilizing gel shift analysis and DNase I footprinting. Previous studies have shown that the PDGF A chain is transcribed in human fetal kidney, and overexpression of this gene has been described in several Wilms' tumors. Thus these studies suggest that lack of WT1 repressor activity may lead to the deregulation of A chain expression, further contributing to the tumorigenic pathway in Wilms' tumors.

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THE HEPATIC GROWTH RESPONSE

Rebecca A. Taub, M.D., *Associate Investigator*

The liver constitutes one of the few normally quiescent tissues in the adult body that has the capacity to regenerate. As a result, it provides a unique multicellular, physiologically normal system in which to study the mitogenic response of epithelial cells. In the rat, following a 70% hepatectomy, the cells in the remaining intact lobes of the liver rapidly resume proliferation. They initiate the first round of DNA synthesis within 12 to 16 hours postsurgery and continue to traverse the cell cycle until the liver regains its initial mass in about 10 days, whereupon they again become quiescent.

Although it is composed mainly of hepatocytes, the liver has a complex, multicellular architecture, implying that intercellular communications must exist during regeneration. Multiple factors, including circulating hormones, growth factors, and nervous input, participate in the regulation of this response, but the actual mechanism remains incompletely understood.

To begin to comprehend the control of liver regeneration, Dr. Taub and her colleagues identified immediate-early growth response genes in regenerating liver and insulin-treated H35 cells, a minimal-deviation hepatoma cell line that has many properties of regenerating liver. Of the 70 total and 41 novel immediate-early genes the laboratory identified, those that fall into one of four categories are being analyzed in detail: 1) novel transcription factor genes, 2) potential growth factor genes, 3) liver-specific immediate-early genes, and 4) genes with abnormal expression in H35 cells compared to regenerating liver.

The Induction Patterns of 70 Genes Posthepatectomy Define the Temporal Course of Liver Regeneration

While the proteins encoded by immediate-early and delayed-early genes are expected to have important roles during regeneration in regulating progres-

sion through the G_1 phase of the cell cycle, it was surprising to find that many of these "early" genes are expressed for extended periods during the hepatic growth response. Dr. Taub's laboratory has defined several patterns of expression of immediate-early, delayed-early, and liver-specific genes during a 9-day period posthepatectomy.

One pattern of induction parallels the major growth period of the liver that ends at 60–72 hours posthepatectomy. A second pattern has two peaks coincident with the first and second G_1 phases of the two hepatic cell cycles. A third group, which includes liver-specific genes such as *C/EBPα*, shows maximal expression after the growth period.

Although the peak in DNA synthesis in nonparenchymal cells occurs 24 hours later than in hepatocytes, most of the genes studied demonstrate similar induction in both cell types. This suggests that the G_0-G_1 transition occurs simultaneously in all cells in the liver, but that the G_1 phase of nonparenchymal cells may be relatively prolonged.

Through these analyses Dr. Taub and her colleagues have been able to define the temporal boundary between proliferation and return to quiescence in the posthepatectomy liver.

PRL-1, a Novel Type of Nuclear/Cytoplasmic Protein-Tyrosine-Phosphatase Induced in the Growth Response

Control of the phosphorylation state of cellular proteins is critical for normal cell growth. Immediate-early genes also play an important role in cell growth regulation. One of the immediate-early genes that the laboratory identified, *PRL-1*, is induced in mitogen-stimulated cells and regenerating liver but is constitutively expressed in insulin-treated rat H35 hepatoma cells that show normal induction of most immediate-early genes.

Sequence analysis revealed that *PRL-1* encodes a novel 19-kDa protein that contains the eight–amino acid active site of the consensus protein-tyrosine-phosphatase (PTPase). PRL-1 has no homology to other PTPases outside this domain, leading to the conclusion that it is a member of a new class of PTPases. Bacterially expressed PRL-1 is able to dephosphorylate the phosphotyrosine analogue *p*-nitrophenylphosphate (PNPP) but shows no activity for serine-threonine phosphorylated substrates. PRL-1 itself contains consensus sites for tyrosine phosphorylation, is phosphorylated *in vitro* by *src* kinase, and shows some ability to autodephosphorylate. Antibody localization studies indicate that PRL-1 is present in both cytoplasmic and nuclear fractions of cells.

Among the products of immediate-early genes, PRL-1 is the first PTPase to be identified and, like the cdc25 family of nuclear PTPases, may have an important role in cell cycle regulation in the mammalian growth response.

Transcriptional Regulators Active During Liver Regeneration: LRF-1 and Rat IκBα

Understanding the basis for transcriptional responses during liver regeneration is of major importance in understanding cell growth. Dr. Taub and her colleagues have identified a novel, abundant immediate-early gene that encodes a 21-kDa leucine zipper–containing protein designated LRF-1 (liver regeneration factor). In regenerating liver, LRF-1, JunB, c-Jun, and c-Fos among Jun/Fos/LRF-1 family members are induced posthepatectomy. In liver cells, a high level of c-Fos/c-Jun, c-Fos/JunB, LRF-1/c-Jun, and LRF-1/JunB complexes are present for several hours after the G_0/ G_1 transition, and the relative level of LRF-1/JunB complexes increases during G_1.

Dr. Taub and her colleagues find dramatic differences in promoter-specific activation by LRF-1 and c-Fos–containing complexes. LRF-1 in combination with either Jun protein strongly activates a cAMP response element (CRE)-containing promoter that c-Fos/Jun does not activate. LRF-1/c-Jun, c-Fos/ c-Jun, and c-Fos/JunB activate specific AP-1 and ATF site-containing promoters, and in contrast, LRF-1/ JunB potently represses c-Fos/c-Jun–mediated activation of these promoters.

Repression is dependent on a region in LRF-1 that includes amino acids 40–84 and the basic/leucine zipper domain and, similarly, on a region of JunB that includes amino acids 186–257 and the basic/ leucine zipper domain. As the relative level of LRF-1/JunB complexes increases posthepatectomy, c-Fos/Jun-mediated ATF and AP-1 site activation is likely to decrease with simultaneous transcriptional activation of the many liver-specific genes whose promoters contain CRE sites. Thus, through complex interactions between LRF-1, JunB, c-Jun, and c-Fos, control of delayed gene expression may be established for extended times during the G_1 phase of hepatic growth.

Dr. Taub and her colleagues found that a highly induced immediate-early gene in regenerating liver encodes RL/IF-1 (regenerating liver inhibitory factor), is the rat homologue of human MAD-3 and probably of chicken pp40, and has now been designated rat IκBα. IκBα has IκB activity of broad specificity in that it inhibits the binding to κB sites of p50/p65 NF-κB, c-Rel/p50, and RelB/p50, but not p50 homodimeric NF-κB. Although IκB is a cytoplas-

mic inhibitor of NF-κB that prevents nuclear translocation of NF-κB, some forms of IκB have been found in the nucleus.

Given that some other proteins with notch-like repeats are transcription factors, Dr. Taub wondered whether a nuclear form of IκBα could itself be a transcriptional activator. The group found that Gal4-IκBα fusions strongly transactivate a Gal4 site-containing promoter in 3T3 fibroblasts. The IκBα domain responsible for this transactivation is not the acidic domain of IκBα, but the notch-like repeat domain that has been implicated in the formation of protein-protein interactions.

The subcellular localization of overexpressed IκBα was found to be predominantly cytoplasmic in serum-deprived cells and nuclear in serum-stimulated cells. Thus IκBα appears to have the capacity to enter the nucleus under some cellular conditions. Using double antibody immunoprecipitations, Dr. Taub and her colleagues found that cellular IκBα in proliferating cells is associated with p65 NF-κB in the cytoplasm of cells. However, when fused to the Gal4 DNA-binding domain, some IκBα translocates to the nucleus while associated with p65 NF-κB, thus accounting for its ability to transactivate the Gal4 promoter.

Like IκBα, several members of the NF-κB and rel family of transcription factors are immediate-early genes in regenerating liver and mitogen-treated cells. Dr. Taub and her colleagues examined changes in κB site-binding activity during liver regeneration and discovered a rapidly induced, κB site-binding complex, which was designated PHF (posthepatectomy factor). PHF is induced over 1,000-fold within minutes posthepatectomy in a protein synthesis–independent manner, with peak activity at 30 min, and is not induced by sham operation.

Although the relationship of PHF to other κB site-binding proteins has not been completely characterized, a change in mobility of PHF complexes between 30 min and 3 h posthepatectomy suggests that the composition of the complexes changes. Early PHF complexes do not interact strongly with anti-p50 NF-κB antibodies, but PHF complexes present later (3–5 h posthepatectomy) react strongly, suggesting that they contain a p50 NF-κB subunit.

Because PHF is induced immediately posthepatectomy in the absence of de novo protein synthesis, it could have a role in the regulation of immediate-early genes in regenerating liver.

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THE MOLECULAR BASIS OF METAMORPHOSIS IN *DROSOPHILA*

Carl S. Thummel, Ph.D., *Assistant Investigator*

Pulses of the steroid hormone ecdysone trigger the major postembryonic transitions during the development of the fruit fly *Drosophila melanogaster*. The most dramatic of these transformations occurs at the end of larval development, when a high-titer pulse of ecdysone signals the onset of terminal differentiation into the adult fly. This metamorphosis is accompanied by dramatic changes in gene expression that can be visualized as changes in the puffing patterns of the giant salivary gland polytene chromosomes. Less than 10 "early" puffs form rapidly in direct response to ecdysone. As shown by

Dr. Michael Ashburner and his colleagues, these puffs appear to encode regulatory proteins that both repress their own expression and induce the formation of more than 100 secondary-response "late" puffs. Dr. Thummel's laboratory is studying this genetic regulatory hierarchy at the molecular level.

By isolating and characterizing the ecdysone-inducible genes encoded within the early puff loci, and determining which genes they regulate and how they mediate this control, Dr. Thummel's laboratory hopes to clarify how ecdysone effects the developmental changes associated with metamorphosis. In a broader sense this project provides a model system for characterizing the role of steroid hormones in regulating gene expression as well as addressing the question of how regulatory hierarchies are controlled during development.

Much of Dr. Thummel's research effort is focused on defining the regulation and function of early gene expression. Three early puff loci located at positions 2B5, 74EF, and 75B in the polytene chromosomes have been characterized at the molecular level. The genes corresponding to these puffs are designated the *Broad-Complex* (*BR-C*), *E74*, and *E75*, respectively. In addition, the ecdysone receptor, *EcR*, has been isolated and characterized in Dr. David Hogness's laboratory (Stanford University). These four genes share several features in common. First, they are unusually long for *Drosophila*, spanning 60–100 kb of DNA. Second, they contain multiple nested promoters that are directly activated by ecdysone. Finally, they all encode site-specific DNA-binding proteins, consistent with their proposed regulatory functions. Dr. Thummel's laboratory has shown that the unusual lengths of these genes, combined with their induction by different threshold ecdysone concentrations, contribute to their precise temporal regulation during development.

The control of *E74* transcription typifies the early genetic response to ecdysone. This gene encodes two transcripts, designated *E74A* and *E74B*, from unique promoters. *E74A* transcription is induced several orders of magnitude as a direct response to ecdysone and is subsequently repressed by ecdysone-induced proteins, as predicted for an early response. It is transcribed at a rate of ~1.1 kb/min. This rate measurement, combined with the 60-kb length of the *E74A* unit, accounts for most of the 1-h delay seen between the time ecdysone activates the *E74A* promoter and the appearance of cytoplasmic *E74A* mRNA. Thus the unusual length of the *E74A* transcription unit has a regulatory function, acting as a timer that delays the synthesis of its encoded gene product.

In contrast, *E74B* is designed to be expressed rapidly upon hormonal stimulation. The *E74B* promoter is activated by an ~20-fold lower ecdysone concentration than that required for *E74A* induction. In addition, the *E74B* transcription unit is one-third the length of *E74A*'s, leading to only a 20-min transcriptional delay time. These two factors determine that *E74B* expression will always precede that of *E74A* in response to an ecdysone pulse. Furthermore, *E74B* transcription is repressed as *E74A* is induced, leading to an ecdysone-regulated switch in the expression of these two DNA-binding proteins.

Similar mechanisms control the timing of *E75*, *BR-C*, and *EcR* transcription. For example, the *E75* gene consists of three nested promoters that direct the synthesis of 20-, 50-, and 100-kb primary transcripts. At high ecdysone concentrations, the mRNAs derived from these transcripts appear in an order that is consistent with the lengths of their transcription units. Furthermore, the regulation of each early promoter, with one exception (*E75C*), can be accounted for by its being activated at one of two critical threshold ecdysone concentrations, similar to the two concentrations required for *E74A* and *E74B* induction. These activating ecdysone concentrations are consistent with the temporal order of early gene induction during third instar larval development, suggesting that a gradual increase in ecdysone titer triggers the sequential activation of each early mRNA, leading up to the onset of metamorphosis. Thus the regulatory effects of ecdysone are not dependent solely upon its peak concentrations. Rather, the profile of the hormone pulse contains critical temporal information that is transduced into waves of DNA-binding proteins that could act in a combinatorial fashion to control the timing of subsequent steps in the regulatory hierarchy.

Genetic studies have revealed that this precise sequential induction of early gene expression is critical for an appropriate regulatory response to the hormone. Dr. Thummel's laboratory, in collaboration with Dr. Greg Guild (University of Pennsylvania), has shown that mutations in the *BR-C* early gene result in reduced ecdysone induction of some *E74* and *E75* mRNAs. *BR-C* is expressed before these *E74* and *E75* target promoters are activated, suggesting that the *BR-C* proteins form part of the transcriptional machinery that is required for subsequent *E74* and *E75* induction. Genetic studies of *E74A* in Dr. Thummel's laboratory have shown that the later induction of this mRNA, just preceding puparium formation, has no effect on early gene activity but rather plays a key role in timing late gene induction. In the absence of *E74A* function, many late puffs are either reduced or absent. Dr. Thum-

mel's laboratory has also shown that the *E74A* protein binds to these affected puffs, indicating that it can directly control their activity. Studies are currently under way to define better the regulatory function of *E74A* at the molecular level.

In addition, efforts are under way in Dr. Thummel's laboratory to isolate more regulatory genes that are controlled by ecdysone. It is hoped that these studies will provide further insights into the molecular circuitry involved in the transduction of the ecdysone signal at the onset of metamorphosis in *Drosophila*.

Dr. Thummel is also Assistant Professor of Human Genetics at the University of Utah School of Medicine.

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MOLECULAR GENETIC ANALYSIS OF EARLY DEVELOPMENT

Shirley M. Tilghman, Ph.D., *Investigator*

Parental Imprinting of the Mouse *H19* Gene

Dr. Tilghman's laboratory is studying the mechanisms that underlie the appropriate development of the mammalian embryo. In mammals, development requires the contribution of both the maternal and paternal genomes, a consequence of the fact that a small number of autosomal genes are inherited from their parents in differentially active forms. Two examples of such genes are the insulin-like growth factor II gene (*Igf2*) and *H19*, which lie within 90 kbp of DNA on the distal end of mouse chromosome 7. *Igf2* encodes a growth factor required for embryonic development and is expressed almost exclusively from the paternal chromosome, while the *H19* gene is expressed exclusively from the maternal chromosome. *H19* is an unusual gene, in that it encodes one of the most abundant products of RNA polymerase II in the mouse embryo yet does not appear to encode a protein product.

The close physical linkage of these genes in both mice and humans was established by pulsed-field gel electrophoresis and large DNA cloning in yeast artificial chromosome (YAC) vectors. In addition, analysis revealed that the two genes are expressed in an identical pattern throughout most of development. The one exception to this rule occurs in the choroid plexus and leptomeninges, where both genes are initially expressed. Shortly before birth the *H19* gene is silenced and *Igf2* is no longer imprinted.

The foregoing led to a model that proposes that the imprinting of *Igf2* and *H19* is mediated by a competition in cis for common regulatory elements. The common regulatory elements would explain their very similar patterns of expression during embryogenesis. The competition is set up by epigenetic markings on one or both of the chromosomes, presumably placed there during gametogenesis, the only time when the chromosomes are apart and can be differentially modified. The marking(s) would act to favor transcription of *Igf2* on the paternal chromosome and *H19* on the maternal chromosome. The model can accommodate either a single mark on only one chromosome, or two different marks on the two chromosomes. In the first instance, the chromosome without the mark would transcribe whichever gene is the better competitor for the regulatory elements. The mark could then act either positively or negatively to facilitate transcription of the weaker promoter on the other chromosome.

Zonal Expression of α-Fetoprotein Transgenes in Adult Mice

The developmental regulation of the α-fetoprotein (AFP) gene in liver results in high-level expression in the fetus, followed by dramatic transcriptional repression after birth. The postnatal repression can be perturbed *in vivo* by deleting a region of DNA between −250 bp and −838 bp from

the transcriptional start site. When the distribution of transgene transcripts in liver was examined using *in situ* hybridization, it was shown that they were nonuniform in the liver acinus. Hepatocytes surrounding the central veins expressed high levels of minigene transcripts, while hepatocytes in the intermediate and periportal areas contained few if any transcripts. These results indicate that the complete repression of the AFP gene is a complex process, in that repression in the pericentral hepatocytes is solely dependent upon the presence of the −250- to −838-bp negative-regulatory domain, while the intermediate zone and periportal hepatocytes require an additional element(s).

Genetic and Physical Mapping of the *Fused* Gene

The *Fused* (*Fu*) locus on mouse chromosome 17 was originally identified by spontaneous dominant mutations that result in kinky-tailed mice. *Fu* homozygous mice die at midgestation, as the result of overgrowth of neuroectoderm. In preparation for cloning of *Fu*, the laboratory completed the analysis of a 1,000-animal backcross between Fu^{Ki} mice and *Mus spretus*, designed to localize *Fu* within 0.1 cM, or ∼100–200 kb of DNA. The cross was typed for *Fu*, *tufted* (a recessive phenotypic marker that maps near *Fu*), and four tightly linked DNA markers.

This cross identified the closest linked marker as *Hba-ps4*, a pseudogene of α-globin. Only a single recombination event had occurred between *Fu* and *Hba-ps4* in the 1,000 animals, indicating that they should be within 100–200 kb of each other. Seven overlapping YACs encompassing ∼600 kb of DNA have been isolated using *Hba-ps4* as an entry point. When the farthest ends of this contig were mapped in the backcross, no recombination between them was observed. Thus the region around *Fu* has a low frequency of recombination.

Genetic and Phenotypic Analysis of *piebald*

The spotting of *piebald* is a result of a defect in the development of a subset of the neural crest cells that gives rise to pigment-producing melanocytes. These mice also develop megacolon, the result of the failure of another neural crest derivative, enteric ganglia, to develop appropriately. There are many alleles of *piebald*, thanks to its inclusion as a scorable mutation in a large-scale mutagenesis program,

conducted over many years by Drs. William and Leane Russell at the Oak Ridge National Laboratory. By using cellular markers specific to the neural crest cells leading to melanocytes and/or enteric ganglia, the laboratory established that the *piebald* defect is apparent as early as day 13.5 of embryogenesis, when the neural crest cells are migrating away from the neural tube.

A 500-animal backcross between *piebald* mice and *Mus castaneus* has been generated and analyzed for DNA markers on chromosome 14. One of these markers has been mapped to within 0.5 cM of *piebald*, a distance feasible for chromosome walking. The markers have been used to demonstrate the high degree of heterogeneity in the sizes of the deletions in 13 alleles of *piebald* obtained from Oak Ridge, most of which are embryonic lethals when homozygous.

Dr. Tilghman is also Howard A. Prior Professor of the Life Sciences in the Molecular Biology Department at Princeton University and Adjunct Professor of Biochemistry at the University of Medicine and Dentistry of New Jersey, Robert Wood Johnson Medical School.

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MECHANISM OF TRANSCRIPTIONAL REGULATION IN ANIMAL CELLS

ROBERT TJIAN, PH.D., *Investigator*

Dr. Tjian has focused his research on the specific protein-DNA and protein-protein interactions that regulate the synthesis of RNA in animal cells. The recent molecular cloning and structure-function characterization of DNA-binding transcription factors carried out by Dr. Tjian and his colleagues have helped identify novel protein structural motifs responsible for DNA recognition, protein contact, and transcriptional activation. These studies have, in turn, allowed Dr. Tjian to address the mechanism of action of promoter-specific transcription factors.

In particular, he has obtained evidence for a new class of transcription factors, called coactivators, that seem to function as an intermediary between upstream sequence-specific regulatory elements and the basal-level transcriptional apparatus. This line of investigation, in turn, led Dr. Tjian and his colleagues to the discovery that the TATA-binding protein (TBP) plays a central role in assembling an initiation complex, not only at RNA polymerase II (pol II) promoters but also at RNA pol I transcription. Their finding that TBP is associated with different promoter-selective factors called TAFs (TBP-associated factors) revealed an underlying and unifying principle that appears to govern transcription by all three classes of RNA polymerase. This unexpected discovery significantly altered current views concerning the role of TBP and the mechanism that mediates promoter specificity and transcriptional regulation in animal cells.

Isolating and Characterizing Basal Transcription Factors

The TATA-binding protein, TBP, plays a central role in the initiation of eukaryotic mRNA synthesis. Dr. Tjian recently isolated the human and *Drosophila* cDNA clones for this factor. He and his collaborators have also succeeded in isolating cDNAs encoding a second essential basal transcription factor, TFIIE. Human cDNA clones for both the 56- and 34-kDa subunits of TFIIE were isolated. Using recombinant proteins purified from *Escherichia coli*, they found that both these subunits are required to form a stable preinitiation complex on a basal promoter with TBP, TFIIA, TFIIB, TFIIF, and RNA pol II. Reconstituted transcription reactions establish that both the 56- and 34-kDa subunits of TFIIE are essential for basal-level as well as Sp1-activated transcription *in vitro*.

Analysis of their predicted amino acid sequences reveal several intriguing structural motifs. The 56-kDa subunit sequence includes a zinc finger homology that may interact with DNA, an amphipathic α helix that may define a protein-protein interaction domain, and a protein kinase consensus sequence. The 34-kDa subunit contains a region that resembles an ATP-binding motif. These similarities may provide insights into the role of TFIIE in transcription initiation.

Upstream Activation by Transcription Factor Sp1

The process of transcriptional activation in eukaryotes by site-specific DNA-binding proteins is a key step in gene regulation. When the appropriate trans-activators are assembled at the promoter, they are thought to help direct RNA polymerase to initiate transcription. The mechanism by which sequence-specific transcriptional regulators act to influence rates of mRNA initiation remains largely unknown. Dr. Tjian and his colleagues examined the properties of four distinct activator domains of the human transcription factor Sp1. *In vivo* transient cotransfection assays with Sp1 showed that templates bearing multiple Sp1 sites activated transcription with a high degree of synergism. However, there was no evidence of cooperative binding of Sp1 to adjacent sites.

Using deletion mutants of Sp1, the group determined that the glutamine-rich activation domains A and B and the previously uncharacterized carboxyl-terminal domain D were all required for Sp1 to activate transcription synergistically. Gel-shift, DNase footprinting, and chemical crosslinking experiments revealed a strong correlation between the ability of Sp1 mutants to form homomultimeric complexes and to activate transcription synergistically when bound to multiple sites. The group also examined the process of superactivation, in which a molecule of Sp1 tethered to DNA via its zinc fingers could be transcriptionally enhanced by interacting directly with fingerless Sp1 molecules. The domains involved in superactivation appeared to be a subset of those necessary to achieve synergistic activation. These findings suggested that different domains of Sp1 carry out distinct functions and that the formation of multimeric complexes may direct synergism and superactivation.

In an effort to understand how Sp1 could activate a TATA-less promoter, Dr. Tjian and his colleagues previously addressed the factor requirement at a promoter containing only Sp1-binding sites and an

initiator element. They described a novel activity that was present in the HeLa TFIID fraction but distinct from TBP and the Sp1 coactivator and that was only required for activation of a TATA-less promoter by Sp1. In the presence of promoter-bound Sp1, this so-called tethering factor apparently functioned as a substitute for the TATA box in transcription assays *in vitro*. These observations led to the proposal that Sp1 plays an essential role in assembling the basal initiation factors, possibly by anchoring TBP to the TATA-less template via the tethering factor.

Purification of the TAF/TBP Complex

Recently substantial progress has been made in characterizing the nature and complexity of coactivators in both human and *Drosophila* cells. Chromatographic studies indicate that the endogenous TFIID consists of a multisubunit complex containing the TBP, coactivators, and other associated factors. A fraction containing the coactivator activity was separated from the endogenous TBP after disrupting the tightly associated complex with urea. The two separated components, when added together with the Gal4-Pro activator, restore activated levels of transcription. Immunoaffinity purification of the TFIID complex identifies several polypeptides specifically associated with the endogenous TBP, some or all of which function as coactivators when reconstituted with activators. The isolated coactivators also mediate activation by a chimeric glutamine-rich activator derived from Sp1, but not the Gal4-VP16 activator, suggesting distinct factor requirements for different types of transcriptional regulators.

Using a combination of high-resolution chromatography and antibody affinity chromatography, Dr. Tjian and his group recently succeeded in purifying to homogeneity several of the TAFs. The isolated polypeptides were subjected to proteolysis, the amino acid sequences of purified peptides were determined, and DNA probes derived from the protein sequences were generated. In addition, monoclonal antibodies directed against individual TAFs were isolated and used to probe λgt11 cDNA libraries. By means of these two strategies, six of the TAFs have recently been cloned. Their structure and function are being studied.

Characterization of the RNA Polymerase I Factor SL1 Revealed Unexpected Unifying Mechanisms for Transcription in Eukaryotes

The study of transcriptional initiation unraveled elegant but complex sets of biochemical interactions among sequence-specific DNA-binding proteins, promoter/enhancer elements, and the basal transcriptional apparatus. However, the molecular interactions that took place between the DNA-binding factors and components of the basal apparatus that included RNA polymerase and a variety of accessory transcription factors remained elusive. Transcription by RNA pol I offered some unique advantages in studying the mechanism of promoter recognition and activation.

In particular, Dr. Tjian and his colleagues ascertained that only one type of promoter in each species but at least two transcription factors—the promoter selectivity factor (SL1) and upstream binding factor (UBF)—are necessary to direct accurate and promoter-specific transcription of rRNA genes in animal cells. UBF was the only RNA pol I transcription factor that was necessary for initial promoter binding. The second essential factor, SL1, did not bind specifically to the human promoter by itself. However, when both UBF and SL1 were present, a strong cooperative DNA-binding complex with an extended DNA-binding region was formed at the human rRNA promoter that was critical for transcriptional initiation.

The SL1-UBF complex was reminiscent of the situation that occurs between site-specific upstream enhancer factors and potential interactions with components of the basal RNA pol II transcriptional machinery. Was there, perhaps, a common mechanistic link between the RNA pol I initiation factor SL1 and components of the initiation complex utilized by RNA pol II?

It was previously known that TBP and multiple TAFs are required for regulated transcriptional initiation by RNA pol II. This year Dr. Tjian and his group reported the biochemical properties of the RNA pol I promoter selectivity factor, SL1, and its relationship to TBP. Column chromatography and glycerol gradient sedimentation indicated that a subpopulation of TBP copurified with SL1 activity. Antibodies directed against TBP efficiently depleted SL1 transcriptional activity, which was restored with the SL1 fraction but not purified TBP. Thus TBP was necessary but not sufficient to complement SL1 activity.

Analysis of purified SL1 revealed a complex containing TBP and three distinct TAFs. Purified TAFs reconstituted with recombinant TBP complemented SL1 activity, and this demonstrated that TBP plus novel associated factors are integral components of SL1. These findings suggest that TBP might be a universal transcription factor and that the TBP-TAF arrangement provides a unifying mechanism for promoter recognition in animal cells.

Dr. Tjian is also Professor of Molecular and Cell Biology at the University of California, Berkeley, and Adjunct Professor of Biochemistry and Biophysics at the University of California, San Francisco.

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GENETIC DEFECTS IN THE METABOLIC PATHWAYS INTERCONNECTING THE UREA AND TRICARBOXYLIC ACID CYCLES

David L. Valle, M.D., *Investigator*

The general theme of Dr. Valle's research is human genetic diseases, particularly disorders of amino acid metabolism, retinal degenerations, and inborn errors of peroxisome biogenesis. During the past year he has continued to work on an inborn error of amino acid metabolism that produces a retinal degeneration, gyrate atrophy of the choroid and retina (GA). This rare, autosomal recessive, blinding disorder is characterized biochemically by ornithine accumulation and results from inherited defects in ornithine-δ-aminotransferase (OAT).

As an extension of this research, Dr. Valle and his colleagues are cloning and characterizing other genes whose expression is important for the normal function of retinal photoreceptors. They are also studying a group of closely related genetic disorders characterized by abnormal peroxisome formation. Peroxisomes are spherical, single membrane-bound organelles containing >40 matrix enzymes that are involved in a variety of oxidative and synthetic processes. Zellweger syndrome (ZS), a genetically heterogeneous, autosomal recessive, lethal disorder, is the clinical paradigm for inborn errors of peroxisomal biogenesis.

Ornithine-δ-Aminotransferase

Analysis of the OAT mutations in GA. Dr. Valle and his colleagues have now identified 37 mutant OAT alleles. Together with 10 mutant OAT alleles reported by other groups, these mutations account for about two-thirds of all the possible 174 abnormal alleles in the probands of the 87 GA pedigrees being analyzed in Dr. Valle's laboratory.

One recently recognized OAT allele of particular interest, A226V, may be important for understanding the molecular basis of vitamin-responsive inborn errors. The mutant enzyme responds to high concentrations of pyridoxal phosphate both *in vivo* and *in vitro*. The amino acid substitution is four residues upstream of E230, which is predicted on the basis of homology to another pyridoxal phosphate–dependent enzyme to interact with N1 of the pyridoxal phosphate molecule. A second recently recognized OAT allele was detected in several members of a large Italian kindred and results from the deletion of a 5'-untranslated sequence, leaving the translated portion of the OAT mRNA intact. Despite this, the mutation appears to block translation

and reduce the steady-state level of the mutant mRNA. Expression studies to investigate these effects directly are in progress.

Part of the project described above was supported by a grant from the National Eye Institute, National Institutes of Health.

Cloning of genes for enzymes metabolically related to OAT. Seeking a better understanding of coordinate regulation of OAT with other, metabolically related enzymes, Kristianne Dougherty, a human genetics graduate student in Dr. Valle's laboratory, cloned a cDNA for Δ^1-pyrroline-5-carboxylate (P5C) reductase. This enzyme catalyzes reduction of P5C, the product of the OAT reaction, to proline. This human cDNA was cloned by functional complementation of the corresponding mutant in *Saccharomyces cerevisiae*. Utilizing the P5C reductase cDNA, Dougherty cloned and characterized its structural gene (P5CR.1) and a second gene that appears to encode a closely related P5C reductase (P5CR.2) with 86% amino acid identity with P5CR.1. Studies are under way to investigate the possibility that the qualitative biochemical differences in P5C reductase activity of various tissues may result from tissue-specific differences in the expression of P5CR.1 and P5CR.2.

Regional expression of OAT in liver. In collaboration with Dr. James Darnell (Rockefeller University), Dr. Valle and his co-workers discovered that expression of OAT in human and rat liver is localized to hepatocytes surrounding the central vein of the hepatic lobule in a pattern identical to that of glutamine synthetase. In contrast, expression of ornithine transcarbamylase and the other enzymes of the urea cycle is limited to periportal hepatocytes. Exclusion of OAT from periportal hepatocytes is hypothesized to allow for accumulation of ornithine necessary for ureagenesis. Transgenic experiments with an OAT minigene driven by a periportal-specific promoter are in progress to test this hypothesis.

Animal models for GA. Why is the retina uniquely sensitive to the metabolic disturbances in GA? To answer this question, Tao Wang, a graduate student in human genetics working with Dr. Valle, is attempting to produce a mouse model for GA by gene knockout. Initial studies utilizing a targeting construct containing nonisogenic murine OAT gene sequences did not yield homologous recombination events. Therefore, Wang has cloned OAT gene sequences from the AB-1 embryonic stem cell line and is producing targeting constructs containing these isogenic sequences.

Cloning and Analysis of Genes Whose Expression Is Limited to Photoreceptors

Carol Freund, another human genetics graduate student, has cloned the human homologue of recoverin, a protein that mediates the Ca^{2+}-dependent stimulation of photoreceptor guanylate cyclase and plays a role in recovery of the photoreceptor to the dark-adapted state. Freund has cloned and sequenced the human recoverin cDNA; cloned, mapped, and characterized the recoverin structural gene; and identified several intragenic polymorphisms useful for linkage studies. Currently she is investigating the possible involvement of recoverin in a variety of retinal degenerations.

Reverse Genetic Approach to Cloning Retinal Degeneration Genes Mapping in or Near Xp11.2

Dr. Michael Geraghty, an HHMI-supported postdoctoral fellow in the laboratory, has developed yeast artificial chromosome (YAC) contigs covering the OATL1 and OATL2 loci in the Xp11.2 region. These loci each contain several OAT-related pseudogenes and are separated by 1–2 mb. Dr. Geraghty used a YAC from OATL1 to screen a human retinal cDNA library and cloned seven cDNAs whose genes map back to this region. Four of these originate from single-copy genes in Xp11.2; the others have considerable repetitive sequences and appear to represent false positives. The possible role of these genes in various X-linked retinal degenerations that map to this region of the X chromosome is under investigation.

Molecular Studies of Inborn Errors of Peroxisome Biogenesis

Drs. Jutta Gärtner and Emily Germaine-Lee, postdoctoral fellows in the laboratory, are testing the hypothesis that genes encoding peroxisome-specific integral membrane proteins may be involved in disorders of peroxisomal biogenesis such as ZS. Study of these genes also should enhance understanding of how normal peroxisomes are assembled and function. Initial studies have focused on PMP70, which is a member of the ATP-binding cassette (ABC) transporter protein family. In 35 probands with ZS, representing five complementation groups (provided by Dr. Hugo Moser), Dr. Gärtner found two complementation group 1 patients with mutations in PMP70. One mutation is a simple missense allele, G17D; the other results in aberrant splicing. The splicing mutation results in replacement of the normal carboxyl-terminal 25 amino acids of PMP70 with an unrelated sequence of 23

amino acids. This deletes several carboxyl-terminal residues that are highly conserved in ABC transporter proteins. Functional analyses of these alleles are in progress.

Dr. Valle is also Professor of Pediatrics with joint appointments in Medicine, Molecular Biology and Genetics, Biology, and Ophthalmology at the Johns Hopkins University School of Medicine.

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GENETIC DISEASES OF THE HUMAN X CHROMOSOME

STEPHEN T. WARREN, PH.D., *Associate Investigator*

Fragile X Syndrome

Since the discovery of the *FMR-1* (fragile X mental retardation 1) gene by Dr. Warren and his collaborators in early 1991, his laboratory has continued to investigate the multitude of interesting aspects of this unique locus. A mutation of this locus, associated with a chromosomal fragile site at Xq27.3, is responsible for the fragile X syndrome, the most common inherited cause of mental retardation.

Fragile X syndrome is an X-linked dominant disorder with reduced penetrance in both sexes (80% in males and 30% in females). It is unusual in that penetrance is variable within families, depending on the relationship to nonpenetrant transmitting males (NTMs). Male siblings of NTMs have a <9% risk of mental retardation, yet grandsons of NTMs carry a 40% risk. The risk increases up to normal Mendelian ratios (i.e., 50% affected sons) by passage through female meioses.

This phenomenon, termed the Sherman paradox, is equivalent to genetic anticipation where increasing clinical involvement and/or earlier age of onset,

rather than risk of penetrance, increases through generations. As discussed below, continued investigation of the fragile X mutation by Dr. Warren and his collaborators has demonstrated the molecular basis of the Sherman paradox and begun to illuminate the molecular mechanism(s) of the disease.

Within the 5'-untranslated region of the *FMR-1* transcript is a CGG repeat that is normally polymorphic (mean length of 29). In penetrant patients with fragile X syndrome, this repeat is massively expanded beyond 200 repeats, often exceeding 1,000 triplets. When the repeat length exceeds approximately 230 triplets, the *FMR-1* gene, including its promoter and the repeat itself, becomes heavily methylated. This methylation leads to transcriptional silencing of the gene. The subsequent lack of *FMR-1* gene product, whose function is currently unknown, presumably leads to the phenotype associated with the fragile X syndrome (mental retardation with characteristic facies and macroorchidism in affected males).

Carrier individuals, who themselves are unaffected, have intermediate levels of CGG-repeat expansion from 52 to 200 triplets, and alleles of this size in fragile X pedigrees exhibit marked meiotic instability. Each time the fragile X chromosome transmits from carrier to offspring, it changes to a repeat length different from the parental chromosome (usually larger) and distinct from that of siblings. Thus the mutation rate of the fragile X allele is 1. Female carriers, but significantly not male carriers, have a chance with each meiosis of transmitting not just a slightly different allele size to a child, but rather a massively expanded repeat allele leading to the fragile X syndrome.

Dr. Warren and Dr. David L. Nelson (Baylor College of Medicine) with Dr. C. Thomas Caskey (HHMI, Baylor College of Medicine) established that the repeat length of the fragile X chromosome of carrier mothers influences this probability of massive expansion, such that a carrier with 60 repeats has approximately an 8% chance of having an affected son, while another carrier mother with 100 repeats has a 50% risk. Since NTMs (who always have less than 200 repeats) have mothers with less than 90 repeats, their siblings are less likely to be affected. As the mutant chromosome is transmitted, sometimes for a number of generations, it can continually increase in size, such that descendant females have an increasing risk of having an affected child. These data therefore resolve the Sherman paradox and provide a molecular basis for the reduced penetrance in this disorder.

To determine the normal function of the *FMR-1* gene product and deduce how its deficiency leads to fragile X syndrome, Dr. Warren's laboratory has cloned and sequenced the entire gene from the mouse. In turn, the murine *fmr-1* cDNA was used to identify both the yeast and *Caenorhabditis elegans* homologues of this highly conserved gene. The murine gene has 95% amino acid identity to the human. It contains a CGG repeat in the 5'-untranslated region, although smaller than human, with nine triplets. The gene is expressed in similar tissues in both human and mouse, primarily the brain and testes, as expected from the clinical picture.

Working with Dr. David Housman (Massachusetts Institute of Technology), Dr. Warren and his colleagues conducted *in situ* hybridization studies in various mouse developmental stages. Widespread expression of *fmr-1* was found throughout fetal development. Analysis of adult brain localized *fmr-1* expression to the cerebellum and hippocampus, with intermediate expression in the cerebral cortex selectively exhibited in neuronal rather than glial cells. Examination of testes was suggestive of Sertoli cell expression.

Soon after the discovery of the fragile X mutation, two other disorders, myotonic dystrophy and spinal-bulbar muscular atrophy, were similarly found to be due to expansions of transcribed triplet repeats. To identify other such loci and determine the extent of such genes, Dr. Warren's laboratory screened human cDNA libraries with repeat probes and searched databases for cDNAs with triplet repeats. Forty such genes were identified, and 14 have been characterized to date. Five were found to contain transcribed triplet repeats, which are normally highly polymorphic, a characteristic of the three-repeat expansion disorders. Four of these genes were similar to *FMR-1*, containing CGG repeats in 5'-untranslated regions. Several were of previously determined function, and examination of potentially related disorders is ongoing.

Emery-Dreifuss Muscular Dystrophy

Dr. Warren's laboratory has continued the refinement of the map position of Emery-Dreifuss muscular dystrophy (EDMD), an X-linked dystrophy distinct from the Duchenne or Becker forms. Using an exceptionally large family in north Georgia, more than 100 meioses have been analyzed for linkage. When compiled with other data, the results place the EDMD locus between the Xq28 loci, encoding the color vision genes, and coagulation factor 8.

In collaboration with Dr. Jed Gorlin (Harvard Medical School), the gene for actin-binding protein 280 (ABP 280) was placed within this map interval

of <500 kb. ABP 280 is an attractive candidate gene for EDMD, as it is a structural protein with an amino portion quite similar to the dystrophin protein responsible for DMD and BMD. Similar to dystrophin, much of the central protein is composed of a repeating motif. Expression of the ABP 280 protein is ubiquitous among tissues, including muscle and heart, and various isoforms exist. Current efforts involve the identification of muscle-specific ABP 280 exons and the search for mutations in EDMD families.

Xq28 Mapping

Efforts continue to fine-map the terminal band of the human X chromosome Xq28, which lies immediately distal to the fragile X site. In the past year, an Xq28 physical map of 9 Mb was constructed with Drs. Annemarie Poustka (Heidelberg) and Hans Lehrach (Imperial Cancer Research Fund, London). Highly polymorphic loci (DXS455 and DXS548) were also cloned and mapped to this region, as was the human biglycans gene. Further refinement of the map positions of X-linked hydrocephalus and nephrogenic diabetes insipidus in Xq28 was accomplished as well. (The projects in this section were supported by a grant from the National Institutes of Health.)

Dr. Warren is also Associate Professor of Biochemistry and of Pediatrics at Emory University School of Medicine.

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THE *MyoD* GENE FAMILY: A NODAL POINT DURING SPECIFICATION OF MUSCLE CELL LINEAGE

Harold M. Weintraub, M.D., Ph.D. *Investigator*

The *MyoD* gene converts many differentiated cell types into muscle. The protein MyoD, a member of the basic helix-loop-helix (bHLH) domain, contains a 68–amino acid segment that is necessary and sufficient for myogenesis. It binds cooperatively to muscle-specific enhancers and activates transcription. The helix-loop-helix motif is responsible for dimerization, and control of MyoD activity depends on its dimerization partner.

MyoD senses and integrates many facets of the cell state. The gene is expressed only in skeletal muscle cells and their precursors; in nonmuscle cells it is repressed by specific genes. The protein activates its own transcription, perhaps stabilizing commitment to myogenesis. Despite this seemingly overwhelming evidence that *MyoD* is crucial for myogenesis in vertebrates, recent experiments with Drs. Michael Krause and Andrew Fire show that zygotic deletions of *MyoD* in worms result in embryos that retain the capacity to activate muscle cell differentiation, presumably because there are redundant or alternative pathways for myogenic activation.

Muscle-Specific Transcriptional Activation by MyoD

Dr. Weintraub and his colleagues have been focusing on the mechanism by which MyoD activates transcription. A major paradox is that other bHLH proteins similar to MyoD fail to activate myogenesis. For example, E12 binds to the same sequence as MyoD, but it is expressed in all cell types. Previous experiments showed that when the 13–amino acid basic region of the ubiquitously expressed bHLH gene *E12* replaces the corresponding basic region of MyoD, the resulting MyoD-E12Basic chimeric protein could bind specifically to muscle-specific enhancers and form dimers with E12, but cannot activate a cotransfected reporter gene or convert $10T^1/_2$ cells to muscle. Back mutation of this chimeric protein (with the corresponding residues in MyoD) reestablishes activation. A specific alanine is involved in increasing DNA binding, and a specific threonine is required for activation. These results define certain residues in the basic region of MyoD as necessary for myogenic activation. When these residues are "recognized" as being bound to the proper DNA-binding sites, subsequent myogenic activation can occur.

By replacing the *MyoD* basic region and the adjacent four-residue junction region with helix 1 into the corresponding region of *E12*, Dr. Weintraub and his colleagues have shown that this small section of MyoD (21 amino acids) is sufficient for myogenesis. Further work suggests that only three residues, A_{114}, T_{115}, and K_{124}, are uniquely critical for "recognition factor" function and subsequent activation of myogenic gene transcription.

Control of MyoD Activity

A variety of transforming agents inhibit myogenic differentiation, including a variety of growth factors, oncogenes (*src*, *ras*, *fos*, *jun*, *fps*, *erb*A, *myc*, and *E1A*), and chemical agents such as butyrate and phorbol esters. Most of these reagents can inactivate the expressed MyoD protein; in addition, several (such as *ras* and *fos*) also inhibit *MyoD* transcription. Whether this is a secondary effect due to an inhibition of the autoactivation function of MyoD protein or a more direct inhibition of *MyoD* transcription remains to be determined. Rhabdomyosarcoma cells (derived from tumors of patients who harbor a genetic predisposition to myogenic tumors) differentiate poorly but express MyoD, suggesting that loss of anti-oncogene activity at the rhabdomyosarcoma locus can also impinge on MyoD action. The specific pathway by which each of these oncogenes, anti-oncogenes, and growth factors inhibits myogenesis provides a potential clue to how MyoD might integrate information coming from many aspects of cellular function.

Recently, in collaboration with the laboratory of Dr. Inder Verma (Salk Institute), Dr. Weintraub and his colleagues have found that the leucine zipper region of the protein expressed by the *jun* oncogene actually binds to the helix-loop-helix region of MyoD, both *in vivo* and *in vitro*. Similarly, assays for recognition factor for MyoD activation show that such a factor, which is missing in rhabdomyosarcoma cell lines, can be provided in trans by fusion with $10T^1/_2$ cells. Possibly, failure to activate myogenesis leads to increased proliferation, and then secondary effects that give rise to rhabdomyosarcomas.

Activation of MyoD During Development

Developmental activation of MyoD is being studied in three organisms: mice, worms, and frogs. In both mice and worms, deletional analysis has identified regulatory sequences upstream of the *MyoD* gene that are important for correct developmental

activation of MyoD. Current efforts focus on identifying trans-acting elements that integrate with these sequences. In worms, several maternal-effect mutants have been isolated by Dr. Jim Priess and his colleagues. These mutant embryos produce excess muscle from the wrong lineage. It is possible that they define elements involved in the segregation of myogenic potential to specific cells during early cleavage stages. In apparent contrast to worms, frogs seem to activate MyoD in all cells of the blastoderm; however, expression is stabilized only in those presumptive mesodermal cells that become induced by vegetal inducing factors such as activin. Frogs also contain maternal MyoD mRNA, which, however, seems not to be crucial for subsequent myogenesis, as its destruction with antisense DNA results in normal muscle gene activation.

The ts41 Chinese Hamster Cell Cycle Mutant

The ts41 mutation of Chinese hamster cells was first isolated and characterized by Drs. Joseph Hirschberg and Menasahe Marcus (1982), who showed that at nonpermissive temperature, cells accumulate up to 16°C equivalents of DNA. Dr. Weintraub and his colleagues have shown that the mutation is recessive and that at nonpermissive temperature cells replicate their genome normally but, instead of going on into G_2, M, and G_1, pass directly into a second S phase. This entry into a second S phase does not require serum, nor is it inhibited by G_2 checkpoints or mitotic inhibitors.

Temperature shift experiments suggest that the ts41 gene product participates in two functions in the cell cycle: entry into mitosis and inhibition of entry into S phase. The ts41 mutation seems to define a new class of cell cycle mutant that couples the sequential events of DNA replication and mitosis. (This research was supported by a grant from the National Institutes of Health.)

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CHARACTERIZATION OF NEWLY ISOLATED DISEASE GENES

RAYMOND L. WHITE, PH.D., *Investigator*

Adenomatous Polyposis Coli

In mid-1991 the gene responsible for familial adenomatous polyposis coli (APC) was identified in Dr. White's laboratory, after a molecular search of the region of human chromosome 5q21 that had been identified by linkage studies in families exhibiting this autosomal dominant disease. Nothing was known about the gene before it was found, but the putative physiological role of *APC* as a tumor suppressor had made it a target of interest in a number of laboratories. Affected members of APC families are predisposed to colon cancer because adenomatous colonic polyps, precursors of both sporadic and inherited carcinomas, usually become numerous in early adulthood. Although APC is a rare disease, an understanding of the causative gene could shed light on the mechanisms leading to common, presumably nonfamilial colon cancers as well.

Identification of new mutations. Several approaches to characterizing *APC* and its mutant forms are under way in Dr. White's laboratory. Dr. Joanna Groden continues to identify mutations in the constitutional DNA of APC patients, by single-strand conformation polymorphism (SSCP) analysis and by DNA sequencing. Nearly all of the mutations in *APC* found to date, here or elsewhere, have consisted of base-pair changes leading to stop codons or of small deletions leading to shifts in the reading frame. Many of them are unique to a single kindred.

Few mutations have been detected in the small

exons at the amino terminal of *APC*; most known changes have occurred in exon 15, the largest and most 3' of the coding regions. However, Lisa Spirio has identified a 4-bp deletion in the third exon of *APC* in a family segregating an attenuated form of polyposis. This is the most 5' mutation observed to date. Ms. Spirio has also identified a highly polymorphic, dinucleotide-repeat marker system, <100 kb away from the *APC* gene, that is contributing to the precision of linkage studies and presymptomatic diagnoses in other families with atypical APC.

Dr. Mark Leppert has shown that the phenotype of the attenuated form of this inherited disease may vary widely within a single kindred, from only one or a few polyps to many. Since the phenotype of individuals with few polyps overlaps with that of many people in the general population, mutations in the APC gene may well define some (perhaps a significant proportion of) individuals in the general population who are at high risk for colon cancer.

Structure of the protein product of APC. Sequencing studies have revealed that *APC* can be transcribed in two different ways—i.e., alternatively spliced—in normal human tissues. The messages encode predicted polypeptides that are 2,742 or 2,843 amino acids long, with no extended sequence homologies to other known proteins. Clear functional motifs are absent. However, the first 900 residues of the gene product contain proline-free blocks containing heptad repeats of hydrophobic residues—a pattern characteristic of proteins that form coiled coils.

Experiments by Dr. Geoffrey Joslyn have shown that the first 55 amino acids of the APC protein, encoded within the first three exons, are sufficient to form the parallel, helical dimer expected of a coiled coil. To achieve this result, Dr. Joslyn chose a system in which fragments of *APC* were tested for their ability to encode a dimerization domain for the cI repressor of phage λ in an *Escherichia coli* expression system. The physical properties of a fusion peptide containing the 55–amino acid segment strongly favor a two-stranded coiled-coil structure for this domain.

The significance of these findings to the abnormal function that leads to APC is speculative at present, but the position of the dimerization domain at the amino terminus of the gene might account for the dominant nature of the inherited disease by permitting a peptide product with a mutation farther downstream to dimerize with the product of the normal allele and "poison" the complex.

Dr. Groden has initiated experiments designed to test the ability of nonmutant *APC* to revert colon carcinoma cells to normal morphology. Cell lines have been transfected with a full-length cDNA carried on a puromycin-selectable retroviral vector, and the outcome is now being characterized.

Neurofibromatosis 1

Ongoing investigations of the neurofibromatosis 1 gene (*NF1*), a tumor suppressor that was identified in Dr. White's laboratory two years ago, and its protein product, neurofibromin, are designed to clarify normal function and to explain how mutation leads to development of neurofibromas. Like APC, NF1 is associated with an increased risk of malignancy in carriers of a mutant allele. Gangfeng Xu is studying the promoter region upstream of *NF1* for clues to expression of this gene and has constructed a full-length cDNA that is available to other investigators for a variety of biochemical studies.

Also in this laboratory, Ying Li and Dr. Richard Cawthon have demonstrated that mutations in *NF1* occurring in somatic cells can contribute to development of sporadic cancers, including tumors not associated with neurofibromatosis. (These NF1 studies have received partial support from the National Neurofibromatosis Foundation and the National Institutes of Health.)

Studies of Other Diseases

In collaboration with clinicians in Utah and elsewhere, studies are under way to map the causative genes in a variety of autosomal diseases. Several dozen new markers for chromosome 8 have been developed in an effort to map more precisely a gene on this chromosome that carries mutations responsible for multiple exostoses. During the past year Dr. Louis Ptacek, in collaboration with the group under Dr. Leppert's direction, showed that mutations in an adult skeletal muscle sodium-channel gene are responsible for a disabling muscle weakness, hypercalcemic periodic paralysis.

Genome Project

Dr. White directs numerous activities of the Utah Genome Center, which is part of the Human Genome Project supported by the National Institutes of Health. His laboratory is developing highly polymorphic new DNA markers that will permit construction of high-density linkage maps of human chromosomes. Unlike traditional RFLPs (restriction fragment length polymorphisms), the new markers need not be maintained as clones, because they are reproduced by means of the PCR (polymerase chain reaction) and automated sequencing. These sequence-tagged sites (STSs) contain tandemly re-

peated dinucleotide or tetranucleotide sequences and unique flanking sequences. Because their sequences are known, STS markers can bridge the gaps between genetic and physical maps of chromosomes.

New genetic maps for chromosomes 13, 17, and 20, containing STSs and other markers, have been submitted for publication. Physical mapping in chromosomal regions of interest is also under way. For example, Dr. Hans Albertsen has identified more than 30 YACs (yeast artificial chromosomes) from a region of chromosome 17 believed to contain an inherited gene for premenopausal breast cancer.

Dr. White is also Thomas D. Dee II Professor of Human Genetics and Adjunct Professor of Cellular, Viral, and Molecular Biology at the University of Utah School of Medicine.

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MOLECULAR HEMATOPOIESIS

DAVID A. WILLIAMS, M.D., *Assistant Investigator*

The aim of research performed in Dr. Williams's laboratory is an understanding of the basic biology of hematopoietic stem cells. Specifically, Dr. Williams investigates the relationships between primitive hematopoietic cells (termed stem cells) and other cells located in the blood-forming environment. The studies are designed to define more clearly the cell-cell interactions, growth stimulatory proteins, and other local environmental controls that are important in the normal and abnormal production of blood cells. The knowledge gained may provide better approaches to growing blood cells in the laboratory and may thereby improve bone marrow transplantation and gene therapy methods.

Gene Transfer Into Hematopoietic Stem Cells

The use of retroviral vectors to effect transduction of hematopoietic stem cells has been associated with several difficulties. Previously, long-lived and reconstituting murine hematopoietic stem cells could only be targeted for gene transfer infrequently. This is still the case in larger animals, such as dogs and monkeys. In addition, the level and stability of expression of the inserted gene in primary hematopoietic cells (cells derived from infected stem cells) *in vivo* has been problematic.

Dr. Williams's laboratory and other investigators have now demonstrated long-lived expression of several genetic sequences in mice following transplantation of bone marrow into which these sequences have been introduced. Molecular analysis has demonstrated that the genetic sequences have entered very primitive stem cells in the marrow that can give rise to blood cells for prolonged periods, probably the lifetime of the transplanted mice.

In an attempt to apply the same technology to human diseases, Dr. Tom Moritz in Dr. Williams's laboratory has been carrying out studies with both monkey and human bone marrow. Along with Drs. David Bodine and Arthur Neinhuis at the National Institutes of Health, Dr. Moritz has been able to demonstrate gene transfer of adenosine deaminase (ADA) into bone marrow cells that are capable of differentiation for prolonged periods in transplanted monkeys.

The deficiency of ADA causes the fatal, inherited disease in children called severe combined immunodeficiency (SCID). Use of retroviral vectors to transfer functional ADA into hematopoietic cells is one approach to genetic therapy of this disease. Although these experiments demonstrate that the ADA gene has been introduced into only a few primitive bone marrow stem cells, the expression of the ADA protein is easily detectable in the blood and bone marrow of the transplanted monkey. The laboratory is continuing these studies in collaboration with the NIH investigators.

In addition, Dr. Moritz has investigated the introduction of genes into human cells. Human umbilical cord blood was previously shown to contain primitive hematopoietic cells that can give rise to all blood cells when transplanted into another human. Thus cord blood is a highly available source for transplantable blood-forming cells. Dr. Moritz has concentrated his studies on determining the efficiency with which genes can be introduced into these cells.

His studies have demonstrated that genes can be introduced into the primitive cells of umbilical cord blood easier than into those of the adult bone marrow. The reason for the difference is not clear and is currently being investigated. This finding, however, may indicate that umbilical cord cells could be used as a source for transplantable hematopoietic cells that contain introduced gene sequences.

Hematopoietic Stem Cell Interactions with Their Microenvironment

The survival and proliferation of hematopoietic stem cells *in vivo* and *in vitro* is dependent on direct interaction of these cells with cells or extracellular matrix proteins comprising the hematopoietic microenvironment in the bone marrow cavity. Various cell types in this complex environment can be immortalized, using recombinant retroviral vectors containing the simian virus 40 large T (SV40 LT) oncogene. This procedure has allowed the detailed analysis of interactions between the stem cells and their microenvironment.

Previous work in Dr. Williams's laboratory had demonstrated that primitive hematopoietic cells that can give rise to all blood lineages adhere to specific proteins within the bone marrow cavity. Dr. Moritz has continued investigations into the role that adhesion of primitive cells to this protein plays in blood cell development. He has used gene transfer via retroviral vectors to study the effect of adhesion on primitive hematopoietic cell proliferation.

In another series of experiments, Dr. Xunxiang Du has performed detailed investigations on the effects of administering a growth factor, interleukin-11 (IL-11), produced by cells in the bone marrow cavity, on blood cell formation in the mouse after radiation- or chemotherapy-induced marrow injury. Radiation and chemotherapy are often used to treat cancer, and bone marrow injury is a serious side effect of these therapies.

Dr. Du has demonstrated that treatment of mice with IL-11 can accelerate the recovery of bone marrow from these toxic side effects and significantly reduce mortality in mice treated with very high doses of radiation and chemotherapy. These beneficial side effects may prove useful in cancer therapies. In addition, Dr. Du has demonstrated that IL-11 regulates the amount of lipid accumulation in the bone marrow cavity.

Dr. Pamela Kooh is taking a similar approach to study the normal function of the growth factor IL-11 in the bone marrow. Using a series of experiments done both *in vitro* and *in vivo*, Dr. Kooh hopes to demonstrate the role of IL-11 in normal bone marrow as well as in fetal blood development.

Dr. Manas Majumdar is investigating the biology of another protein produced by bone marrow cells. The protein, Steel factor, is deficient in certain mice with a genetic form of bone marrow disease. Dr. Majumdar is investigating the importance of the way Steel factor is presented to developing blood cells in the marrow cavity. The presentation of Steel factor is evidently important for human blood cell development in tissue culture systems, as previously shown by Dr. Denis Toksoz in Dr. Williams's laboratory. By studying the mouse, Dr. Majumdar can better explore the role of Steel factor protein *in vivo*.

Dr. Williams is also Associate Professor and Kipp Investigator of Pediatrics and Associate Professor of Medical and Molecular Genetics at Indiana University School of Medicine, Herman B Wells Center for Pediatrics Research, and Associate Professor of Pediatrics at the James Whitcomb Riley Hospital for Children, Indianapolis.

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MOLECULAR GENETICS AND GENE THERAPY FOR METABOLIC DISORDERS

Savio L. C. Woo, Ph.D., *Investigator*

Dr. Woo and his colleagues are interested in studying phenylketonuria (PKU), a genetic disorder that predisposes affected children to develop severe mental retardation. The disorder is secondary to a genetic deficiency of the enzyme hepatic phenylalanine hydroxylase (PAH). It is transmitted as an autosomal recessive trait and has a prevalence of ~1 in 10,000 Caucasians and 1 in 16,500 Orientals.

Molecular Basis and Population Dynamics of PKU

The establishment of multiple RFLP (restriction fragment length polymorphism) sites in the human PAH (hPAH) gene and its use in prenatal diagnosis of PKU have been reported previously. The 3-allelic HindIII RFLP system in the hPAH gene was shown to be caused by an AT-rich (70%) minisatellite region. This region contains various multiples of a 30-bp tandem repeat and is located 3 kb downstream of the final exon of the gene. Characterization of this variable-number tandem repeat (VNTR) region by PCR (polymerase chain reaction) amplification indicated that the previously reported 4.0-kb HindIII allele contains 3 such repeat units, and the 4.4-kb HindIII allele contains 12 repeats. On the other hand, the 4.2-kb HindIII fragment can contain 6–9 copies of this repeat, which permit more detailed analysis of mutant chromosomes bearing a 4.2-kb allele.

Kindred analysis in PKU families demonstrated Mendelian segregation of the VNTR alleles, as well as associations between these alleles and certain major PKU mutations. For example, the R261Q mutation is associated almost exclusively with an allele containing 8 repeats, and the IVS-10 mutation with one containing 7 repeats. The combined use of this VNTR system and the existing RFLP haplotypes increases substantially the performance of prenatal diagnostic tests for PKU. In addition, this VNTR will be most useful in studies concerning the origins and distributions of prevalent PKU mutations in various human populations.

Tissue-Specific Expression of the hPAH Gene

In primates PAH is expressed specifically in the liver. In rodents PAH activity is also present in the kidney, although at a much lower level. A 9-kb genomic DNA fragment flanking the 5′ end of the hPAH gene was fused to the bacterial gene for chloramphenicol acetyltransferase (CAT). The hPAH-CAT minigene was used to generate multiple transgenic mouse lines. In all expressing lines, CAT activity was detected predominantly in the liver and, at much lower levels, in the kidney.

By immunohistochemical staining, CAT expression was localized to hepatocytes and renal epithelial cells, both of which also express the endogenous mouse PAH gene. Furthermore, both the transgene and the endogenous mouse PAH gene were activated at about the same stage of embryonic development in the mouse liver. These results suggest that the 9-kb DNA fragment flanking the 5′ end of the hPAH gene contains all necessary cis-acting elements to direct its tissue-specific expression and development regulation *in vivo*.

The 319-bp region immediately flanking the initiation site of the gene is characterized by the lack of a proximal TATA box and the presence of sequences homologous to GC boxes, CACCC boxes, CCAAT boxes, activator protein 2–binding sites, glucocorticoid, and cAMP response elements. When this short DNA fragment was used to direct the transcription of a reporter gene, specific expression in hepatoma cells was observed. The results suggest that the hPAH gene has a TATA-less promoter regulated by multiple transcription factors and that the system will permit the identification and characterization of mutations in the promoter region of the hPAH gene that cause PKU. (This portion of the work was supported in part by a grant from the National Institute of Child Health and Human Development, National Institutes of Health.)

Somatic Gene Therapy of Hepatic Deficiencies

The construction of a recombinant retrovirus bearing hPAH cDNA under the transcription regulation of a liver-specific promoter was reported previously. The recombinant retrovirus was capable of transducing primary mouse hepatocytes in culture, and hPAH mRNA accumulated in the cells to a level comparable to that present in normal human liver. Dr. William Dove's laboratory at the University of Wisconsin recently created a mouse strain deficient in PAH that has provided an excellent model system for exploring the possibility of its phenotypic correction by hepatic gene therapy. A recombinant retrovirus containing mouse PAH cDNA was constructed and used to transduce hepatocytes isolated from the PAH-deficient mice.

The transduced hepatocytes produced dramatically higher levels of mouse PAH mRNA than did mock-infected hepatocytes. The PAH mRNA was translated efficiently into PAH protein that is capable of converting phenylalanine to tyrosine *in vitro*. These results demonstrate that the PAH-deficient mouse hepatocytes can be readily reconstituted by retroviral-mediated gene transduction. (This portion of the work was supported in part by a grant from the National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health.)

The next step is to develop technologies for the efficient transfer of therapeutic genes into living animals in order to correct a deficient phenotype. The current strategy for hepatic gene therapy involves the isolation of primary hepatocytes from a resected liver lobe and transduction of therapeutic genes into the cultured cells, followed by autologous hepatocellular transplantation. The *ex vivo* approach has been reported in mice, rabbits, and dogs, but is a complex procedure in its entirety. Thus a simple method for direct gene delivery into hepatocytes *in vivo* was developed. The procedure involves partial hepatectomy followed by portal vein infusion of recombinant retroviral vectors.

Histologic analysis of hepatocytes after *in vivo* delivery of a recombinant retrovirus bearing the *Escherichia coli* β-galactosidase gene showed that 1–2% of the parenchymal cells were transduced. Direct hepatic transfer of human α_1-antitrypsin cDNA under the transcriptional direction of the albumin promoter-enhancer led to constitutive expression of the human protein in the sera of mouse recipients at concentrations of 30–1,400 ng/ml for at least 6 months. The experimental animals showed no signs of illness, and histologic analysis of the liver revealed no evidence of pathologic abnormalities. The persistent expression of a foreign gene in the recipient liver suggests that the *in vivo* approach is an attractive alternative to hepatic gene therapy. (This portion of the work was supported in part by a grant from the National Heart, Lung, and Blood Institute, National Institutes of Health.)

Dr. Woo is also Professor of Cell Biology, Molecular Genetics, and Pediatrics at Baylor College of Medicine.

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MOLECULAR GENETIC STUDIES OF BEHAVIOR AND DEVELOPMENT IN *DROSOPHILA*

Michael W. Young, Ph.D., *Investigator*

Studies of Biological Rhythms

Certain behaviors in *Drosophila melanogaster* have a circadian rhythm. Locomotor activity and eclosion both occur rhythmically with 24-hour periodicities. The rhythms must reflect action of an endogenous clock, as persistent environmental cycling is not required to maintain the rhythms. The laboratory has recently completed a study of the ontogeny of *Drosophila*'s clock(s) controlling circadian behaviors.

Drosophila born and reared in constant darkness and at constant temperature exhibit circadian locomotor activity rhythms as adults. Thus exposure to environmental cycling does not appear to be needed to establish biological rhythmicity. However, the circadian rhythms of the individual flies composing these dark-reared populations have many different phases. The absence of synchrony among flies extends to those commencing development at the same time. The latter results show that a biological clock(s) controlling these rhythms in *Drosophila* begins to function without a developmentally imparted phase.

The phases of rhythms produced by dark-reared flies can be synchronized by supplying light treatments during development. Experiments of this sort have been used to map the earliest detectable function of a *Drosophila* clock(s). Light treatments initiated and ending as early as the developmental transition from embryo to first-instar larva can synchronize adult locomotor activity rhythms. Because the behavior of adult flies is seen to be synchronized by treatments ending in early development, a clock controlling circadian rhythms should function continuously from the time of larval hatching to adulthood.

The flies' circadian rhythms are influenced by mutations of the *period* (*per*) gene. Three mutations were characterized in earlier studies. Two of these, *per*ˢ and *per*ˡ, are associated with single–amino acid substitutions. The first, *per*ˢ, shortens the period to

19 hours, while *per*ˡ lengthens it to 28 hours. The third mutation, *per*°, results in arrhythmic flies and a truncated, presumably functionless protein.

In dosage studies using transgenic *Drosophila* that express different levels of *per* RNA, the laboratory has shown that period length is correlated with abundance of the *per* product. Those flies producing lower levels of *per* RNA have longer-period circadian rhythms. Because *per*ˡ and *per*ˢ mutants produce wild-type levels of *per* RNA, it was thought that the mutants might produce hypoactive and hyperactive *per* proteins, respectively. Dr. Young and his colleagues have been interested in determining if and how a change in *per* protein structure might increase the protein's activity.

Genes carrying new *per* mutations have been produced by *in vitro* mutagenesis and returned to *Drosophila* by P-element–mediated transformation. A wide variety of missense mutations, each altering the same region of ~20 amino acids around the site of the original *per*ˢ mutation, have been characterized. The mutations predominantly generate short-period phenotypes. In many cases double– and triple–amino acid substitutions were generated. In fact, the line with the shortest period rhythm, ~1.5 hours shorter than the original *per*ˢ mutant, was associated with a triple–amino acid substitution.

These results indicate that short-period phenotypes result from loss rather than gain of function in a limited domain of the *per* protein. Possibly this region of the protein suppresses activity of the *per* protein, and therefore plays a regulatory role, in wild-type flies.

The laboratory has been characterizing a new chromosome-linked clock mutation. The *timeless* (*tim*) mutation produces arrhythmia for both eclosion and locomotor activity. In addition to its effects on behavioral rhythms, the *tim* mutation affects expression of *per*. Although the *per* locus is found on the X chromosome, *tim* alters circadian timing of *per* transcription. Since the arrhythmic phenotypes

of *tim* mutants resemble those of *per°* mutants, it is possible that *tim* alters rhythmicity because it affects *per* transcription. It is equally possible that *tim* affects *per* indirectly, by eliminating an upstream biological clock.

Genetic Control of Neuromuscular Development

Now recognized in *Drosophila* are seven zygotically required genes whose absence results in overproduction of neuroblasts at the expense of epidermoblasts. Recently, from collaborative studies with Dr. Thomas P. Maniatis and his colleagues (Harvard University), Dr. Young's laboratory has discovered for these genes a very different role. Each gene is essential for correct patterns of mesodermal development.

Mesodermal expression of all of the neurogenic genes has been observed, beginning at about the time of neuroblast delamination in the embryo. Loss of any of the neurogenic genes results in overproduction of certain mesodermal cells that normally give rise to precursors of muscle. This hyperplasia in the mutants affects at least the somatic mesoderm, and probably occurs at the expense of another mesodermal cell type(s), in a fashion similar to the switching of cell fates in the ectoderm. In some mutants, cell fusions required for formation of multinucleate muscle are also eliminated. Strong effects on differentiation of visceral mesoderm also have been seen. So far there is no indication that these mesodermal effects depend on aberrant development of the associated ectoderm in the mutants.

The results suggest that the seven genes may work together to influence cell fate choices undertaken independently in mesoderm and ectoderm. Rather than provide specific instructions about what a cell's final fate is to be, the products of these genes may allow cells to choose between alternative cell pathways common to many tissue types, with the ultimate fate of the cell determined by the local history of the tissue involved.

In other collaborative work with Dr. José Campos-Ortega (University of Köln), the laboratory has investigated the molecular details underlying genetic interactions between two neurogenic genes, *Notch* and *Delta*. Both genes code for transmembrane proteins containing several extracellular EGF (epidermal growth factor)-like elements. Aberrant development has been linked to single–amino acid substitutions in EGF elements of *Notch* protein, and some of these mutations are genetically suppressed by single–amino acid substitutions in EGF elements of the *Delta* protein. The collaborative studies have shown that these simple substitutions measurably alter interactions between *Notch* and *Delta* proteins expressed on the surfaces of cultured cells. Some of the results suggest that effects on development are due to altered intracellular signaling by the *Notch* protein in response to novel, direct interaction with *Delta*. (The above studies of neuromuscular development have been supported by a grant from the National Institutes of Health.)

Dr. Young is also Professor of Genetics at the Rockefeller University.

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The Immunology Program is one of the original disciplinary areas of the Institute. Studies of the immune system have led to remarkable recent progress in understanding its development, its unique recognition mechanisms, and the means employed to rid the body of invaders or to neutralize potentially harmful effects.

Investigators in the Immunology Program are located at the University of Michigan, the University of Alabama at Birmingham, the Massachusetts Institute of Technology, Children's Hospital in Boston, the University of Texas Southwestern Medical Center at Dallas, the National Jewish Center for Immunology and Respiratory Medicine at Denver, the University of California at Los Angeles and at San Francisco, Yale University, Albert Einstein College of Medicine of Yeshiva University in the Bronx, Rockefeller University, Stanford University, the California Institute of Technology, the University of Washington in Seattle, and Washington University in St. Louis.

Research in the laboratory of Assistant Investigator David G. Schatz, Ph.D. (Yale University) focuses on understanding the process by which the immune system recognizes and neutralizes invading pathogens. Foreign substances are bound by antibodies and the T cell receptors that are found on the surface of immune system cells (lymphocytes). The key to the effectiveness of the immune system is that each of the many millions of lymphocytes expresses a different antibody or T cell receptor. Each of these receptors in turn is encoded by a different gene, and each gene is generated by a gene-shuffling process known as V(D)J recombination. This process cuts and splices DNA within the developing lymphocyte to create the millions of different gene combinations required to encode the receptors. Dr. Schatz's laboratory is interested in identifying the enzyme that carries out V(D)J recombination, determining how this enzyme works, and understanding how the process is regulated during lymphocyte development. Dr. Schatz and his colleagues had previously identified and isolated two genes (the recombination-activating genes, *RAG-1* and *RAG-2*) that appear to encode critical components of this enzyme. His laboratory now hopes to understand why the gene-shuffling process sometimes erroneously generates chromosomal translocations, thereby activating oncogenes and causing leukemia. Finally, they would like to determine if deregulation of, or deficiencies in, the V(D)J recombination process contribute to immunodeficiency or autoimmunity.

T lymphocyte activation is controlled by cell surface proteins that serve as receptors to recognize foreign antigens and bind molecules expressed on other cells in the immune system. The laboratory of Associate Investigator Arthur Weiss, M.D., Ph.D. (University of California, San Francisco) has studied the biochemical mechanisms by which three receptors regulate T cell activation: 1) The T cell antigen receptor recognizes antigen and activates a protein-tyrosine kinase to induce a biochemical cascade of events that contributes to cell activation; 2) CD45, a membrane molecule, has protein-tyrosine phosphatase activity, an enzymatic activity required for antigen receptor signaling function; and 3) CD28 binds to a molecule on antigen-presenting cells and induces a distinct signal transduction pathway required for inducing lymphokine gene expression.

Investigator Susumu Tonegawa, Ph.D. (Massachusetts Institute of Technology) and his colleagues produced several mutant mice, of which each has a defect in an antigen-recognizing molecule expressed on the surface of T lymphocytes (T cell receptors) or in other genes of immunological significance. Using these mice, they dissected the processes by which T lymphocytes differentiate from the immature state to the mature, functional state. They also showed that a particular T lymphocyte subset, $\gamma\delta$ T cells, plays protective roles against bacterial and parasitic infections. The laboratory also produced a mutant mouse deficient in an enzyme (α-calcium/calmodulin-dependent kinase) rich in the synapses of the hippocampus and neocortex of the mammalian brain, and demonstrated that the defect in this single gene causes an impairment in learning the multiple geographical relationships among objects in space.

In the hematopoiesis process, stem cells give rise to different types of mature blood cells, including the white cells of the immune system. Assistant Investigator Stephen T. Smale, Ph.D. (University of California, Los Angeles) and his associates study the molecular mechanisms by which specific genes are turned on and off as white blood cells develop to maturity. They have identified the LyF-1 protein, which appears to turn on several genes in the immature white cells by interacting with the genes' control regions. In addition, they have identified and characterized other features that are unique to the control regions of genes expressed specifically in developing white cells.

Associate Investigator Stanley J. Korsmeyer, M.D.

(Washington University) and his colleagues are concerned with the proto-oncogenes important in lymphocyte development as well as lymphoid malignancies. Highly characteristic translocations between unrelated chromosomes occur in malignant cells of human lymphoma and leukemia. This event places putative cancer genes into a new chromosomal environment. One such gene, *Bcl-2*, isolated from human follicular lymphoma, encodes a novel mitochondrial protein that represses the programmed death of cells. Transgenic mice bearing a deregulated *Bcl-2* gene in their genetic material indicate a normal role for Bcl-2 in maintaining immune responses. Cancer-promoting genes identified in T cell tumors represent regulatory genes diverted from their normal cell type. Transgenic mice that redirect the expression of these genes to the thymus prove that they are oncogenic.

Research performed in the laboratory of Investigator Owen N. Witte, M.D. (University of California, Los Angeles) concerns the interrelated problems of blood cell differentiation and cell growth regulation. This work is focused on two major areas: first, developing techniques to grow and manipulate specific types of blood cells; and second, investigating the function of genes found in human leukemias such as Philadelphia chromosome–positive acute lymphocytic leukemia and chronic myelogenous leukemia. The *ABL* oncogene is changed by rearrangement in these leukemias, resulting in abnormal growth regulation and control. Research in these areas should ultimately increase knowledge of the ways in which oncogene products both regulate and stimulate abnormal cell growth, and aid in an understanding of the mechanisms that govern normal cell growth.

The complement system consists of a group of interactive blood and cellular proteins that recognize and destroy foreign particles. Components of the complement system attach directly to a microbe and then promote its destruction by serving as ligands for receptors on peripheral blood cells. This process must be carefully regulated in order that complement components become attached to foreign material and not self-tissue. A goal of the laboratory of Investigator John P. Atkinson, M.D. (Washington University) is to identify and characterize these regulatory and receptor proteins of the complement system. Special attention has been focused on a multigene family of functionally, structurally, and genetically related, complement regulatory proteins. These molecules are importantly involved in inflammatory processes associated with infections and in autoimmune disease states.

In addition to helping clear and destroy foreign proteins such as those of bacteria and viruses, the complement system plays a fundamental role in the pathogenesis of many human autoimmune diseases, including systemic lupus erythematosus and rheumatoid arthritis. The laboratory of Assistant Investigator V. Michael Holers, M.D. (Washington University) is analyzing the cell membrane proteins that allow the complement system to help regulate the immune response and to clear foreign proteins that complement has attacked. In addition, they are analyzing the roles these proteins play in animal models of human autoimmune diseases. The overall goal is to further an understanding of the complement system and to learn how to modulate its activities in a beneficial manner in autoimmune diseases.

Lymphocytes are the specific mediators of immunity. For lymphocytes to respond to infection or cancer cells anywhere in the body they must move from one part of the body to the next, thus acting as an immunosurveillance network. Lymphocytes move from one organ to another via their expression of homing receptors. The laboratory of Investigator Irving L. Weissman, M.D. (Stanford University) has identified two kinds of homing receptor molecules expressed in lymphocytes that direct their traffic to two kinds of lymphoid organs—lymph nodes (the swollen glands of infection) and the intestine-associated lymphoid organs (tonsils, appendix, and Peyer's patches)—and have isolated the genes that code for these homing receptors. In addition, they have identified one homing receptor that takes lymphocytes to tissues that are infected and/or inflamed. Some lymphocytes that are necessary to eliminate cancer cells, virus-infected cells, or tissue/organ transplants contain within them molecules that mediate cell killing. A set of killer molecules and the genes that encode them have been isolated and the molecular mechanisms of cell killing are under study.

The laboratory of Investigator Roger M. Perlmutter, M.D., Ph.D. (University of Washington) has used molecular genetic strategies to examine signal transduction mechanisms in hematopoietic cells. By expressing novel genes in the lymphocytes of transgenic mice, protein-tyrosine kinases regulating both thymocyte maturation and T cell receptor signaling have been identified. Signaling pathways that direct the appropriate migration of lymphocytes to peripheral immune organs have also been defined. These studies serve to illuminate normal signal transduction mechanisms and provide model systems that should speed development of improved therapies for immunological and neoplastic diseases.

The phosphorylation/dephosphorylation of particular protein-tyrosine residues is a remarkably potent, reversible modification by which cells regulate signal transduction, differentiation, and proliferation. The homeostatic balance controlling the state of phosphorylation is maintained by the coordinate regulation of protein-tyrosine kinases and protein-tyrosine phosphatases. The research interests of Assistant Investigator Matthew L. Thomas, Ph.D. (Washington University) and his colleagues are in understanding how protein-tyrosine phosphatases function to regulate lymphocyte activation and proliferation. Identification and characterization of hematopoietic phosphatases have revealed enzymes potentially involved in regulating various steps of lymphocyte behavior. Functional dissection of the major transmembrane phosphatase of lymphocytes, CD45, has indicated that this enzyme controls antigen-induced activation by regulating the activity of Src-tyrosine kinase family members.

Antibody genes are encoded in pieces in the germline and must be assembled during development of antibody-producing cells. The laboratory of Investigator Frederick W. Alt, Ph.D. (Children's Hospital, Boston) has provided fundamental insights into the mechanism of this gene assembly process and the manner by which its specificity is controlled. Most recently, this group has employed novel approaches to generate mice with several different mutations that affect particular aspects of the development of the immune system. Such mice will be invaluable for ongoing studies of the immune system's function, and some will also serve as models for certain immunodeficiency diseases.

Investigator Mark M. Davis, Ph.D. (Stanford University) and his colleagues are exploring the nature by which a T cell distinguishes self from non-self entities. One aspect of this work is to express specific T cell receptors (the equivalent of antibodies for the T cell arm of the immune system) implanted in mice and observe how they react and develop in a particular genetic background. This work has shown how T cell receptors that might trigger autoimmunity are either removed in the thymus or paralyzed in the peripheral immune organs if they survive thymic selection. Many other T cell receptors are discarded in the thymus because they do not fit that particular animal's complement of histocompatibility molecules. The laboratory is also expressing large quantities of T cell receptors and their target histocompatibility molecules free of the cell membrane in order to reduce T cell recognition to its essential components in a cell-free system.

The analysis of basic properties of CD4 T cells in the laboratory of Investigator Charles A. Janeway, Jr., M.D. (Yale University) has provided information about the ligands, the specific receptors, the co-receptors, and the co-stimulators needed to induce CD4 T cell activation. These technologies and principles are applied to the analysis of disease models. Studies in future years are intended to extend these techniques to problems of autoimmunity and infectious disease, while continuing to expand knowledge of basic immune processes.

The major research interest of Associate Investigator Dan R. Littman, M.D., Ph.D. (University of California, San Francisco) and his colleagues is in the mechanism of differentiation of T lymphocytes. In the thymus, these cells undergo a process of selection that eliminates potentially self-reactive T cells and expands cells that can recognize foreign antigens. This laboratory is studying the functions of surface molecules whose signals contribute to the choice of development of functional helper or cytotoxic cells. One of these molecules, CD4, is necessary, but not sufficient, as a receptor for entry of the human immunodeficiency virus (HIV) into cells. Several approaches are being employed to identify other cellular factors involved in HIV entry. In addition, transgenic mouse systems are being used to elucidate the mechanism of immunopathogenesis of HIV. Information gained from these studies may allow the design of agents that interfere with HIV infection and pathogenesis.

Studies in the laboratory of Associate Investigator H. Kim Bottomly, Ph.D. (Yale University) span the development and peripheral differentiation of subsets of lymphocytes and extend from studies of activation *in vivo* by specific antigens to detailed analysis of specific molecular changes that accompany these differentiative events. The overall goal of these studies is to understand the mechanisms by which the immune system generates the appropriate effector cells to defend against the different forms of pathogens that beset the vertebrate host, as well as those cases in which an inappropriate response leads to a failure in host defense or an attack on self tissues.

Associate Investigator B. Matija Peterlin, M.D. (University of California, San Francisco) and his colleagues study the expression of proteins that help fight infectious diseases and cancer and that prevent organ transplantation. These are the transplantation antigens. Their congenital absence results in severe immunodeficiency and early death. Investigated are signals that direct the synthesis of these transplantation antigens in development and in organs and genes responsible for this rare inherited human im-

munodeficiency syndrome. The laboratory also studies steps that convert latent human immunodeficiency viral infection to active disease. They have found signals that activate viral replication and have elucidated the mechanism of how one viral protein augments these cellular responses. In particular, the Tat protein was found to affect replication of HIV by a new transcriptional mechanism.

Thymus-derived lymphocytes or T cells are an important part of the body's defense against microorganisms. They also are thought to mediate disease development in certain autoimmune diseases and to operate in organ transplant rejection. To understand better how T cells develop under normal circumstances, genes important in T cell function have been cloned and reintroduced into transgenic mice in the laboratory of Associate Investigator Dennis Y.-D. Loh, M.D. (Washington University). Results indicate how T cells learn to discriminate self and non-self markers to maintain the balance of the body's immune system.

T cells recognize the appearance of infections in the body and orchestrate their destruction. In doing this, however, it is essential that T cells do not recognize and turn on the tissues of their own host. Many potentially autoreactive T cells die while they are developing in the thymus. Recent experiments by Investigator Philippa Marrack, Ph.D. (National Jewish Center for Immunology and Respiratory Medicine) and her colleagues show that other such cells die after they have matured when they encounter self elsewhere in the animal. This type of event may affect not only the ability of T cells to destroy their host but also the ability of T cells to attack chronic infections such as herpes or Epstein-Barr virus.

Although T cells of the immune system usually play a critical role in the resistance to microbial infection, some microorganisms produce a superantigen, whose ability to overstimulate T cells helps the pathogen rather than the host. Well-known examples of these proteins are a number of the toxins produced by staphylococcal bacteria, whose activation of very large numbers of T cells can cause the symptoms of food poisoning and toxic shock. These superantigens are under study by Investigator John W. Kappler, Ph.D. (National Jewish Center for Immunology and Respiratory Medicine) and his colleagues. With an understanding of how these proteins interact with the immune system has come the realization that they are much more widespread in the microbial world of bacteria, viruses, and mycoplasma than originally thought. Their disruption of the immune system not only causes obvious acute symptoms but also may have more subtle long-lasting effects, possibly setting in motion the mechanisms of autoimmunity.

Mechanisms of immunological unresponsiveness remain of fundamental interest in understanding the regulation of immune responses. Patients with lepromatous leprosy are immunologically unresponsive to antigens of *Mycobacterium leprae* and unable to control their infection. Studies in the laboratory of Investigator Barry R. Bloom, Ph.D. (Albert Einstein College of Medicine) of the lymphocytes from lesions of this disease and the lymphokines they produce revealed that a subset of T cells, expressing the CD8 surface marker and producing interleukin-4, is responsible for suppressing responses of potentially protective T cells. The related mycobacterium, *M. tuberculosis*, was found to be highly resistant to usual mechanisms of protective killing used by macrophages but uniquely susceptible to reactive nitrogen intermediates, particularly nitric oxide. The study of these infectious diseases is providing insights into basic mechanisms of immunological tolerance and resistance.

The laboratory of Assistant Investigator William R. Jacobs, Jr., Ph.D. (Albert Einstein College of Medicine) has developed the tools to manipulate genetically the slow-growing mycobacteria *M. tuberculosis* and the tuberculosis vaccine, BCG (bacille Calmette-Guérin). By using genetic approaches, it is hoped that significant insights will be gained in understanding the biology of *M. tuberculosis* infections that will be important in developing novel therapies to control this dreaded disease. Another facet of the research involves the cloning of foreign antigens from parasitic pathogens, such as *Leishmania* or *Schistosoma*, into BCG in order to generate recombinant vaccines that might protect against tuberculosis and the pathogen from which the foreign gene is derived.

Cytotoxic T lymphocytes recognize and kill cells that express foreign antigens on their surface. Antigen is presented in the form of short peptide fragments bound to the class I major histocompatibility complex molecules. The laboratory of Investigator Michael J. Bevan, Ph.D. (University of Washington) has been interested in one class I molecule that is specialized to bind and present bacterial peptides to cytotoxic T cells. The class I binding site recognizes the *N*-formylmethionine residue that initiates all bacterial polypeptides.

Investigator Kirsten Fischer Lindahl, Ph.D. (University of Texas Southwestern Medical Center at Dallas) and her colleagues study the function of the nonclassical or medial class I antigens of the mouse major histocompatibility complex. H-2M3 was the

first such molecule that was shown to bind an endogenous peptide and present it on the cell surface. In a collaboration with the laboratory of Dr. Michael Bevan, H-2M3 has now been shown to present a peptide derived from the intracellular parasite *Listeria monocytogenes*. Mice infected with *Listeria* make killer cells that recognize this peptide bound by H-2M3, which therefore plays a role in host defense against bacterial infection. Evidence has also been obtained that another medial class I antigen, Qa-1[b], presents a minimum of four distinct, endogenous peptides.

During a viral infection the immune system kills the virus and destroys infected cells. To do this it relies on one of the host's own proteins, HLA, to bind viral peptides, which are then recognized by T cells. Previous work used x-ray diffraction techniques to get a picture of HLA in atomic detail, and its structure suggested how it binds viral fragments and how T cells recognize it. Assistant Investigator Pamela J. Bjorkman, Ph.D. (California Institute of Technology) and her colleagues are now concentrating on determining the structure of the T cell protein (the T cell receptor) that recognizes the peptide-HLA complex, and on studying the interactions between viral peptides and HLA molecules.

In humans, HLA molecules, the cell surface proteins responsible for graft rejection, are critical for normal immune responses. In a phenomenon called antigen processing, they bind peptides derived from foreign organisms, allowing their recognition by immune T cells; the laboratory of Investigator Peter Cresswell, Ph.D. (Yale University) is studying this activity. Evidence has been obtained that a gene in the HLA complex is required for peptides to bind to the class II subset of HLA molecules. Other genes in the HLA complex are required for the intracellular transport of peptides that associate with class I HLA molecules. In the absence of the latter, peptides generated by a different mechanism, signal sequence proteolysis, were found associated with certain HLA molecules, revealing a new pathway of antigen processing.

Associate Investigator David D. Chaplin, M.D., Ph.D. (Washington University) and his colleagues use yeast artificial chromosome (YAC) clones spanning the human major histocompatibility complex (MHC) as reagents to define the complete gene content of this important complex. Direct nucleic acid hybridization studies have identified a previously unrecognized gene that is expressed exclusively in dermal keratinocytes. Direct cDNA selection strategies have identified additional new HLA genes. Study of these genes may contribute to an under-standing of HLA-linked disease susceptibility. Other work by Dr. Chaplin concerns the immunomodulatory cytokine interleukin-1 (IL-1). A novel cysteine protease, designated the IL-1β convertase, is required for activation of the β isoform of this cytokine. The laboratory has defined the specificity of this protease and has cloned its cDNA. Current studies suggest that the convertase is activated to cleave pro–IL-1β as part of the programmed cell death pathway. Thus IL-1 may either contribute directly in this pathway or may provide systemic signals regarding the occurrence of programmed cell death.

The development of antibody-producing B cells from stem cells in the bone marrow has been a paradigm for studying molecular aspects of mammalian development. The research of Assistant Investigator Sankar Ghosh, Ph.D. (Yale University) and his colleagues is focused on understanding the basis of regulated expression of genes during B cell development. They anticipate that the knowledge gained will ultimately help to determine the causes underlying aberrations that can occur in this developmental pathway, e.g., B cell leukemia and lymphoma. The group is studying in detail how the NF-κB protein functions during B cell development in the production of antibodies. They are attempting to generate transgenic mice where the normal activity of NF-κB is altered, and they hope that such alterations will provide insight into how some malignant disorders of blood cells develop.

Assistant Investigator Michel C. Nussenzweig, M.D., Ph.D. (Rockefeller University) and his colleagues also study B lymphocytes. One important aspect of their development is the regulated assembly of a single antibody gene in each B cell. Allelic exclusion is the mechanism that insures unique clonal specificity in immune responses. Experiments with transgenic mice have shown that exclusion is regulated by a feedback signal from the membrane-anchored form of immunoglobulin protein. Experiments currently under way are aimed at understanding the molecular nature of the feedback signal and how it affects allelic exclusion.

The research in the laboratory of Associate Investigator Craig B. Thompson, M.D. (University of Michigan) focuses on characterizing the molecular events associated with the regulation of lymphoid development and proliferation. Over the past several years the laboratory has made progress in defining how genetic diversity is created in the immunoglobulin genes of developing avian B cells. This work has helped to provide insights into how genetic heterogeneity arises by the process of gene conversion. This group also continues to study gene

regulation during T cell proliferation. They have established a role for the *Ets* gene family in the transcriptional regulation of T cell activation. Studies concerning the physiologic role of the helper T cell–specific receptor CD28 have continued to define its role in regulating immune responses and lymphokine production.

The B cell pathway of differentiation in humans is a focus of study in the laboratory of Investigator Max D. Cooper, M.D. (University of Alabama). Antibodies specific for surrogate light chains and surface immunoglobulin-associated molecules are employed in studies of antigen receptor expression during B cell development and maturation. This information on normal development is used to explore the defects in this differentiation pathway that result in the inherited antibody deficiency diseases of X-linked agammaglobulinemia, common variable immunodeficiency, and IgA deficiency, the last of which occurs at a frequency of approximately 1 in 600 North Americans and Europeans.

Autoimmune diseases such as diabetes mellitus, systemic lupus erythematosus, and myasthenia gravis represent a broad class of disorders caused by immunological attack against normal body components. Assistant Investigator Christopher C. Goodnow, B.V.Sc., Ph.D. (Stanford University) and his colleagues are studying the mechanisms that normally prevent antibodies from being made to normal self components. Antibodies are made by B cells, and during the past year this laboratory has identified key early events that normally lead self-reactive B cells to be either eliminated or inactivated. These advances open up the possibility of understanding the process of self-tolerance in molecular terms and identify steps where the process may break down in autoimmune disease.

The goals of the laboratory of Investigator Richard A. Flavell, Ph.D. (Yale University) are to understand the mechanisms of immune tolerance in which an organism eliminates the ability of lymphocytes to react with its own tissues and instead restricts their immune recognition to foreign antigens. Transgenic mice have been used to assess the factors that upset normal tolerance mechanisms leading to autoimmune disease. Transgenic production of cytokines or co-stimulatory receptors in tissue cells appear to play a role in the induction of an autoimmune state, suggesting that recruitment of cells in an inflammatory response, coupled with their activation, is a key parameter in the induction of autoimmunity. The laboratory has also developed a recombinant vaccine for Lyme disease. This vaccine in now being prepared for clinical testing in human patients, and the group has shown that humans appear to be capable of raising protective antibodies against key components of this vaccine. Moreover, it appears to be efficacious in laboratory experiments where infection is mediated through deer ticks collected in the wild, suggesting that this vaccine may be effective in a natural setting. In other work it was found that the Lyme disease organism appears to use several strategies to evade immune surveillance, one of which appears to be mutation of its outer surface proteins.

Assistant Investigator Donald G. Payan, M.D. (University of California, San Francisco) and his colleagues are elucidating the mechanisms by which certain neuropeptides released into important immunologic microenvironments modulate the responses of distinct leukocyte subpopulations during inflammation. The studies being undertaken explore novel immunologic properties conferred to lymphocytes that express neuropeptide receptors. Additional investigations are aimed at characterizing the protease inhibitor domains of the agrin molecule. Studies on the developing rat demonstrate agrin expression in unique tissues that are undergoing active remodeling, and consequently, agrin may play an important role in neural development.

MECHANISM AND CONTROL OF THE ASSEMBLY OF ANTIGEN-RECEPTOR GENES

FREDERICK W. ALT, PH.D., *Investigator*

Dr. Alt's laboratory is defining molecular aspects of the development of antibody-producing cells, with a focus on the mechanism and control of the genomic recombination events involved in this differentiation process.

Activities Involved in VDJ Recombination

Genes that encode the variable regions of immunoglobulin (Ig) and T cell receptors (TCRs) are assembled from germline variable (V), diversity (D), and joining (J) gene segments during precursor lymphocyte differentiation. All Ig and TCR gene segments are assembled by a common, lymphoid-specific activity referred to as VDJ recombinase.

The VDJ recombination mechanism involves the recognition of conserved recombination sequences (RSs) that flank each germline V, D, or J segment, the introduction of double-stranded breaks at the RS/coding sequence junctions, the potential loss and/or addition of nucleotides at the coding junctions, and polymerization and ligation activities to complete the process—usually resulting in joining of the two participating RSs (RS joins) and in joining of the coding segments (coding joins). By analogy to other systems, the VDJ reaction could be orchestrated by one or two activities that confer specificity, while other events (e.g., ligation) might be effected by ubiquitous cellular activities recruited to function in VDJ recombination.

Tissue-specific activities. The recombination-activating genes 1 and 2 (*RAG-1* and *-2*) were isolated by others, based on an ability to confer VDJ recombination activity to nonlymphoid cells synergistically. To analyze RAG function in normal lymphocytes, Dr. Alt's group used gene-targeted mutation in embryonic stem (ES) cells to generate mice that carry a nonfunctional *RAG-2* gene in their germline. Mice homozygous for the *RAG-2* mutation do not generate mature B or T cells but otherwise appear normal—indicating that *RAG-2* function (and VDJ recombination) is required only for lymphocyte development. Work in the laboratory of Dr. Susumu Tonegawa (HHMI, Massachusetts Institute of Technology) has led to similar conclusions regarding *RAG-1* function.

Rag-2 mutant animals accumulate B and T cell progenitors that have not rearranged their Ig or TCR loci. Pre–B cell lines derived from these mice do not have rearrangements of endogenous antigen receptor loci; however, they rapidly rearrange their Ig heavy-chain locus upon introduction of *RAG-2* expression vectors. Thus B and T cell development in *RAG-2*–deficient mice is blocked due to an inability to initiate VDJ recombination.

Ubiquitously expressed activities. Mice homozygous for the *scid* (severe combined immune deficient) mutation (*Scid* mice) also have impaired ability to generate mature B and T cells. Dr. Alt's laboratory and others demonstrated that pre–B cells in these mice initiate VDJ recombination correctly (including introduction of precise double-stranded breaks) and also form RS joins relatively normally. They are impaired, however, in their ability to form coding joins. Unlike the *RAG-2* mutation, the *scid* mutation is "leaky," in that *Scid* mice develop mature lymphocytes as they age. This leakiness results, at least in part, because liberated coding ends occasionally can be joined by illegitimate recombination to form coding joins. Because RAG-deficient animals cannot initiate VDJ recombination, their defect is not leaky.

Others found that both lymphoid and nonlymphoid cells of *Scid* mice appear to have a defect in DNA repair. Thus the overall phenotype of the *scid* mutation suggests that it may affect a more generally expressed factor recruited to perform one of the terminal events of VDJ recombination.

To elucidate further the potential relationship between VDJ recombination and DNA repair, Dr. Alt's group tested a large number of existing mutant Chinese hamster ovary (CHO) cell lines with defects in either excision repair or double-strand break repair (*dsbr*) for an ability to rearrange introduced VDJ recombination substrates following introduction of constitutive RAG expression vectors. All excision repair mutants showed normal VDJ recombination; however, 3 of 5 tested *dsbr* mutants were markedly impaired in this process. Two (xrs-5 and XR-1) were impaired in an ability to form both coding and RS joins while another (V-3) was impaired primarily in coding but not RS joining (similar, if not identical, to the impairment generated by the murine *scid* defect).

Because each CHO mutant belongs to a different complementation group, their defects are likely encoded by different genes. In this regard, xrs-6 and XR-1 lines that had reverted to normal their DNA repair defect were isolated by others via introduction of specific human chromosomes. These lines also reverted to a normal ability to undergo VDJ recombination. Together the findings indicate that

DNA repair and VDJ recombination share certain components.

Attempts are now being made (in collaboration with Drs. Penny Jeggo and Tom Stamato) to isolate genes encoding activities that complement the CHO mutations. Elucidation of such activities should yield insights into VDJ recombination and, perhaps, into certain human diseases that affect both DNA repair and lymphocyte development.

Control of VDJ Recombination

Regulation of which Ig or TCR gene segments are assembled in particular cells and stages within lymphoid lineages is effected by modulating accessibility of substrate gene segments to the common VDJ recombinase. Dr. Alt's group has developed a novel approach in which test substrates of VDJ recombination are introduced into ES cells, which are subsequently differentiated into normal lymphocytes in somatic chimeric mice.

Assays for specific rearrangement of the substrates in such ES cell–derived lymphocytes demonstrated that several different Ig and TCR transcriptional enhancer elements can also serve to target associated variable-region gene segments for VDJ recombination. To test directly the role of enhancer elements in targeting the recombination of endogenous gene segments, Dr. Alt's group replaced one copy of the Ig heavy-chain intronic enhancer element in ES cells with an expressed neo^r gene without modifying any sequences directly involved in VDJ recombination. These ES cells were used to generate somatic chimeric mice, and ES cell–derived pre–B cell lines were established. Analyses of these cell lines demonstrated that this enhancer replacement resulted in a cis-acting block in the assembly of endogenous Ig heavy-chain variable-region genes.

Dr. Alt's group is currently employing "hit-and-run" gene-targeted mutational approaches to eliminate, mutate, or replace more precisely endogenous transcriptional regulatory elements to study their role in control of VDJ recombination. They are also continuing to employ VDJ recombinase-inducible cell lines that they have recently generated for use in the context of transfection approaches aimed at elucidating in detail how specific motifs within particular enhancer or promoter elements confer accessibility to linked V, D, and J segments for VDJ recombination. (The studies described in this paragraph were also supported by grants from the National Institutes of Health.)

Novel Animal Models to Study Development of the Immune System

Dr. Alt's group has developed a novel ES cell–based approach for rapidly assessing the role of par-

ticular genes or DNA segments in the development or function of lymphocytes. Blastocysts derived from Rag-2–deficient mice are injected with either normal or mutant ES cells and used to generate somatic chimeric mice. Somatic chimeras derived from Rag-2–deficient blastocysts injected with normal ES cells develop mature lymphocytes, all of which are ES cell derived. Therefore it is possible to mutate both copies of any gene that does not affect ES cell viability and test for its role in lymphocyte development by this method.

For example, the group has assayed blastocysts injected with ES cells in which both copies of the Ig heavy-chain locus were disrupted in such a way that it was impossible to form functional heavy-chain genes. The resulting somatic chimeric mice form normal T cells but not B cells. Dr. Alt's group is now employing this approach to test the function of a variety of different lymphocyte-specific and more generally expressed genes (e.g., myc family genes) in the context of lymphoid development.

Dr. Alt is also Professor of Genetics and Pediatrics at the Children's Hospital, Boston, and Harvard University Medical School.

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COMPLEMENT SYSTEM

John P. Atkinson, M.D., *Investigator*

The complement (C) system helps the host prevent infections. It is part of an innate immune system with the capacity to recognize nonself (the alternative pathway) and promote the inflammatory response. It is also a potent effector pathway for antibody (the classical pathway).

When the C system is activated by the binding of antibody to antigen, or by other means, C proteins, particularly an activated fragment of C3, bind covalently to antigens as part of the normal immune response. Such binding also occurs to self tissue in disease states, as part of a pathologic (autoimmune) response. Deposited C3 fragments serve as ligands for C receptors that facilitate the elimination of materials to which C3 is bound.

Regulatory proteins in plasma and on autologous tissue prevent the inappropriate activation and amplification of the C system by controlling this critical step of C3 deposition. As a result, these regulators inhibit C activation in plasma or on self tissue and allow C to amplify only on a foreign target.

Regulators of Complement Activation (RCA)

The RCA is a multigene family of C receptor and regulatory proteins. The members of this family are related functionally (the proteins bind to C components attached to targets), genetically (the genes are located in a tight cluster on the long arm of chromosome 1), and structurally (each protein is largely composed of a tandem array of 60–amino acid, cysteine-rich, repeating modules). Analyses of these proteins have provided insights into the mechanisms whereby C recognizes and destroys microbes while at the same time protecting self tissue. Manipulation of the C regulatory proteins has important implications for tumor, transplantation, and reproductive immunology, as well as autoimmunity.

The human RCA gene cluster has been characterized with yeast artificial chromosomes (YACs). Members of this family include decay-accelerating factor (DAF), complement receptors 1 and 2 (CR1 and CR2), membrane cofactor protein (MCP), C4-binding protein (C4bp), and factor H. Although

the data confirmed many points previously elucidated, the finer resolution of YAC mapping allowed the discovery and/or localization of partial gene duplications, the determination of gene orientations, and the measurement of gaps between known genes. Nine overlapping YACs that encompass a genomic region of 800 kb, encoding four RCA genes and three gene-like elements, were identified.

The encoded genes and two of the gene-like elements shared the same orientation and were ordered (5′ to 3′) DAF, CR2, CR1, MCP-like, CR1-like, and MCP. A C4bp-like region lies upstream from DAF. MCP-like, a new genetic element, was discovered and found to be homologous to the 5′ portion of the MCP gene. Two large gaps of 85 kb (between CR2 and DAF) and 110 kb (between DAF and the C4bp-like element) could carry additional RCA genes.

The arrangement of CR1, MCP-like, CR1-like, and MCP, in that order, strongly suggests that this region was generated by a single duplication of neighboring CR1/CR1-like and MCP/MCP-like forerunners. The RCA YACs will now serve as a convenient DNA source for the subcloning and further characterization of this region.

C3b/C4b receptor, or complement receptor type 1 (CR1, CD35). CR1 is expressed on most peripheral blood cells, including erythrocytes, where it is a critical player in the processing of immune complexes. The Knops, McCoy, Swain-Langley, and York antigens were identified as being on CR1. The relationship between CR1 expression and the reactivity of the CR1-related blood group antigens with their specific antibodies was assessed.

Red blood cells (RBCs) from donors of selected phenotypes were tested by hemagglutination, using two monoclonal antibodies to CR1 and the antisera to the specific blood groups. Monoclonal antibodies (mAbs) 3D9 and E11 required ~250 and ~400 CR1/RBC to obtain a positive reaction. Agglutination of antigen-positive cells by human polyclonal antisera was also related to the CR1/RBC. Thus cells expressing 20–100 CR1/RBC were negative and included the previously designated null phenotypes for this collection, cells expressing 100–150 were weak or negative, and those expressing >200 were usually positive. These data provide an explanation for previously puzzling serologic characteristics of the CR1-related blood group antigen system.

Membrane cofactor protein (MCP or CD46). MCP is a C regulatory protein that is widely expressed on human cells and cell lines. It binds C3b and C4b and functions as a cofactor for their degradation. Because of this, MCP is postulated to protect autologous cells from C-mediated injury.

Human MCP was shown to protect transfected rodent cells from human C-mediated lysis. Furthermore, MCP inhibited C3b deposition in a dose-dependent fashion and inhibited lysis of the mouse cells expressing it. MCP did not inhibit lysis on bystander cells. These results demonstrate the protective role of MCP for the cell on which it is expressed.

The predominant structural motif of MCP is the four short consensus repeats (SCRs). These domains are responsible for ligand binding in other C regulatory proteins. SCR-deletion mutants were constructed to determine which of the four SCRs of MCP contribute to ligand-binding and cofactor activity. Analysis of the deletion mutants indicated that the third and fourth SCRs were important for both ligand-binding and cofactor activity of C3b and C4b. In addition, the same SCRs were required for binding of an mAb that inhibits MCP's function.

The deleted mutant of SCR-2 bound but lacked cofactor activity for C3b. It did not bind or possess cofactor activity for C4b. Deletion of the first (amino-terminal) SCR had a minimal effect on C3b-binding and cofactor activity but reduced the efficiency of C4b binding. The results identify the SCRs of MCP that contribute to ligand-binding and cofactor activity. The data also suggest the presence of distinguishable C3b- and C4b-binding sites and provide evidence that C3b binding is not always sufficient for cofactor activity.

An MCP-like molecule on the inner acrosomal membrane of human spermatozoa has been characterized. Three mAbs and a rabbit polyclonal antibody against MCP recognized the sperm protein. On SDS-PAGE (sodium dodecyl sulfate–polyacrylamide gel electrophoresis), sperm MCP migrated as a single band with a molecular mass of 38 and 44 kDa under nonreducing and reducing conditions, respectively. The molecular mass is 10–20 kDa less than the forms of MCP expressed on other cells.

In contrast to the MCP of other cells, the electrophoretic pattern, by one- and two-dimensional gel analysis, and the isoelectric point profile (4.5 to 5.0) of the sperm protein were similar among multiple individuals. Furthermore, digestion with endoglycosidases did not alter either the molecular mass or the isoelectric point of the protein, suggesting that it is a poorly or nonglycosylated form of MCP. The solubilized sperm protein bound C3b and possessed cofactor activity for factor I–mediated cleavage of C3b. An mAb that blocks the regulatory function of MCP inhibited the cofactor activity of the sperm lysate.

Thus the sperm protein is an antigenic and functional homologue of MCP but has the distinct structural feature of a lower molecular mass secondary to

a lack of glycosylation. MCP may play an essential role in the survival of the acrosome-reacted spermatozoa by modulating C activation in the female genital tract.

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MICHAEL J. BEVAN, PH.D., *Investigator*

Cytotoxic T lymphocytes (CTLs) recognize foreign antigens as peptide fragments bound to the cell membrane class I molecules that are encoded in the major histocompatibility complex (MHC). These peptides bind the class I molecule in the endoplasmic reticulum and are an essential stabilizing feature of the final structure. The peptides can come from any protein present within the cell—normal self proteins or an antigenic viral protein, for example. The so-called classical class I MHC molecules—HLA-A, -B, and -C in humans and H-2K, -D, and -L in mice—are extremely polymorphic, and each allelic form binds a different range of peptides.

In addition to these well-known class I MHC molecules that act as peptide presenters, there are a large number of so-called nonclassical, or class Ib, MHC molecules with much more limited polymorphism. When an activated CTL recognizes a cell expressing a foreign peptide, it can rapidly lyse the cell and release cytokines. In this way CTLs provide a surveillance mechanism against virally and bacterially infected cells and possibly tumor cells expressing aberrant genes.

Responses to Intracellular Bacteria

A small number of bacteria, such as *Listeria*, *Shigella*, and *Rickettsia*, gain access to the cytoplasm of cells, where they are able to replicate and hide from the antibody-mediated and phagocytic arms of the immune system. *Listeria monocytogenes* is a ubiquitous food-borne pathogen. Dr. Bevan and his colleagues have been studying the class I MHC-restricted response to peptides derived from *Listeria* and presented on the surface with class I MHC molecules. A year ago they reported the classical presentation of a *Listeria* peptide to CTLs by a conventional class I molecule. During the course of these studies, they became aware that some of the peptides present on the surface of *Listeria*-infected cells could be recognized in a non-MHC–restricted way. This implied that the peptides were not being presented by the classical MHC molecules but perhaps by the class Ib molecules. In collaboration with Dr. Kirsten Fischer Lindahl (HHMI, University of Texas Southwestern Medical Center at Dallas), they were able to show that the relatively nonpolymorphic class Ib gene *H-2M3* was indeed responsible for presenting this *Listeria* peptide to CTL.

The peptide itself is resistant to aminopeptidase digestion, implying that it may have a blocked amino terminus, and its interaction with the class I molecule is competed by peptides that begin with *N*-formylmethionine. All bacterial proteins are initiated with *N*-formylmethionine, whereas nuclear eukaryotic proteins initiate with methionine. Within eukaryotes only 13 genes, those encoded by mitochondria, initiate with *N*-formylmethionine, reflecting their bacterial origin. It seems likely, therefore, that the nonclassical class I molecule H-2M3 has evolved to bind *N*-formylmethionine peptides derived from intracellular bacteria and to present them to specific CTLs. This is the first time that the nonclassical class I molecules have been shown to be recognized during the course of an infection. It is not yet known whether the other nonclassical class I molecules also have a similarly unique role in antigen presentation to T lymphocytes.

How Do CTLs Protect Against Intracellular Bacteria?

Most bacteria live extracellularly, and 99% of them are removed by phagocytosis, aided in some cases by opsonizing antibody. Most researchers would have predicted that the protective T cell response to intracellular bacteria would be heavily dependent on the interferon-γ (IFN-γ) release by CTLs serving to activate macrophages for phagocytosis. It came as a surprise, therefore, to discover that CTLs specific for a single nonameric bacterial peptide plus a class I MHC molecule could protect animals in an IFN-γ–independent fashion.

These experiments were done by loading the animals with a neutralizing anti–IFN-γ antibody. In such animals CD4 class II–restricted T cells lose their ability to protect against *Listeria monocytogenes* infection, implying that the IFN-γ–mediated activation of macrophages is very important in this case. However, with class I–restricted CTLs, neutralization of the IFN-γ had no effect. It seems likely that the CTLs work by rapidly lysing infected cells and thus limiting their rate of multiplication. CTLs probably cannot kill bacteria directly, and it may be that neutrophils kill them in the extracellular space following their release from infected cells.

Positive Selection

CTLs and helper T cells are selected in the thymus on the basis of their antigen/MHC-specific receptors. It is a common assumption that cortical epithe-

lial cells expressing class I and class II MHC molecules are responsible for this selection. Heretofore, however, there has been no tumor model for positive selection. In collaboration with Dr. Barbara Knowles (Wistar Institute), Dr. Bevan's laboratory has identified a line of simian virus 40 (SV40)-transformed thymic epithelial cells that, when injected intrathymically, mediates the selection of cytotoxic T cells. Because the antigen-processing and presentation system of this cell appears to be quite conventional, i.e., like that of every other cell in the body, the result implies that the peptide-MHC complexes that mediate positive selection do not differ from those that mediate negative selection. It has been suggested that the positively selecting MHC molecules express a different range of peptides from normal cells, but this no longer seems likely.

The tumor cell line grows well *in vitro* and will be a useful tool to study other facets of positive selection. For example, one can regulate the levels of MHC expressed by this cell and add, or possibly take away, certain other accessory molecules to study their influence on positive selection.

A grant from the National Institute of Allergy and Infectious Diseases provided support for the project described above.

Dr. Bevan is also Professor of Immunology at the University of Washington, Seattle.

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STRUCTURAL STUDIES OF CELL SURFACE MOLECULES INVOLVED IN RECOGNITION BY THE IMMUNE AND NERVOUS SYSTEMS

Pamela J. Bjorkman, Ph.D., *Assistant Investigator*

Dr. Bjorkman and her colleagues are interested in the structure and function of molecules involved in cell surface recognition, particularly those that mediate the immune response. Their approach is three-fold, combining x-ray crystallography to determine three-dimensional structures, molecular biological techniques to produce and modify proteins for crystallization, and biochemistry to study the proteins made.

T Cell Recognition

T cells have a complicated recognition system that includes a membrane-bound receptor (the T cell receptor, or TCR), the multisubunit CD3 molecule, and the accessory or co-receptor molecule CD4 or CD8. A fundamental difference between recognition by T cells and by antibodies is that antibodies bind to soluble antigens, whereas TCRs recog-

nize a fragmented form of the antigen (derived through intracellular processing) bound to a molecule of the major histocompatibility complex (MHC). MHC molecules bind peptides derived from self and foreign proteins during synthesis and transit to the cell surface. In this work the laboratory seeks a molecular understanding of MHC-peptide interactions and TCR recognition of MHC-peptide complexes.

Crystallization of a TCR and its MHC-peptide ligand. In collaboration with Dr. Mark Davis (HHMI, Stanford University), a project has been initiated to crystallize soluble forms of a TCR and the MHC-peptide complex it recognizes. Crystals of 2B4, a well-characterized TCR with specificity for the class II molecule IE^k complexed with a peptide of pigeon cytochrome *c*, have been obtained, as well as crystals of the IE^k–cytochrome *c* peptide com-

plex. The TCR crystals are small (~50 μm^3), and can only be obtained from glycosidase-treated preparations of protein.

The seven potential N-linked glycosylation sites in 2B4 have been altered by site-directed mutagenesis in an attempt to produce homogeneous protein and eventually improve the crystals. COS-7 cells transfected with the modified cDNAs show cell surface expression of each mutant chain, and stable Chinese hamster ovary (CHO) lines expressing the aglycosylated proteins are being established. The IE^k-peptide crystals diffract to ~4.0 Å. To improve the data obtained from these crystals, the use of synchrotron radiation and cryopreservation, two well-established methods for enhancing data quality, is being explored.

Stability studies of MHC molecules with and without peptide. A soluble form of the class I MHC molecule H-2K^d has been expressed in CHO cells at levels up to 100 mg/l. After removal of endogenous peptide, K^d was renatured from separated heavy and light chains. The resulting empty K^d heterodimer was immunologically reactive and structurally similar to a heterodimer renatured in the presence of an appropriate restricted peptide. Overall yields of reassembled empty and peptide-filled K^d are 45–50%, allowing sufficient material for crystallization.

Thermal stability profiles indicate that the two forms of heterodimer differ in their resistance to denaturation by heat, but that a significant portion of the empty class I molecule has a native conformation at physiological temperatures. These data are consistent with the possible existence of empty MHC molecules *in vivo*, which may play a role in immunological processes such as the positive selection of T cells during maturation in the thymus.

Free energies were calculated from a thermodynamic analysis of the stability curves, giving a direct measure of the stabilization of the class I molecule due to peptide binding. The stability assay employed is a novel way of evaluating the binding of peptide to purified MHC molecules and can be used to compare the degree of stabilization conferred by different peptides. Future plans include use of thermal stability measurements to assess the effects of peptide length and composition on stabilization of class I heterodimers. (A grant from the National Institutes of Health provided support for the work described above.)

**Expression and Crystallization
of an Fc Receptor Related to Class I
MHC Molecules**

Fc receptors expressed in the gut of newborn rodents bind to maternal immunoglobulin in milk at pH 6.5 and transport it to the bloodstream of the neonate, where it dissociates at pH 7.4. The intestinal Fc receptor (FcRn) was recently observed to employ the same light chain as class I MHC molecules and to have a heavy chain with significant sequence similarity to heavy chains of MHC. Although FcRn is predicted to share the same type of groove that serves as the MHC peptide-binding site, the immunoglobulin ligand of FcRn is not a peptide but a macromolecule. The structural similarity between these molecules that function so differently in the immune system affords an opportunity to study how evolution has utilized this structural motif for different purposes.

A secreted version of the rat Fc receptor has been expressed in CHO cells, and soluble Fc receptor has been purified with high yields (~40 mg/l) from cell supernatants. In contrast to the structurally related class I MHC molecules, purified FcRn shows upon analysis no endogenously bound peptides. Crystals of soluble FcRn diffract to 2.7 Å at room temperature, and cryopreserved crystals diffract to 2.2 Å. Complexes of FcRn and Fc have been prepared, and the stoichiometry of binding has been determined to be two FcRn molecules per Fc. Crystals of the FcRn-Fc complex have also been obtained, and an analysis is in progress. (This work is supported by the National Institutes of Health.)

**Structural Studies of Surface Recognition
Molecules in the Nervous System**

A number of the molecules found to contain immunoglobulin homology units mediate cell-cell interactions in the nervous system as well as the immune system. In collaboration with Dr. Allan Bieber (Purdue University), a project to crystallize *Drosophila* neuroglian is under way. Neuroglian is a cell adhesion molecule with an extracellular portion consisting of six immunoglobulin-like domains followed by five fibronectin type III repeats. From an expressed version of these repeats, a stable proteolytic fragment corresponding to three repeats has been isolated and crystallized. Native and derivative data from cryopreserved crystals have been collected, and a structure determination is in progress.

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IMMUNITY AND PATHOGENESIS OF THIRD WORLD DISEASES: LEPROSY AND TUBERCULOSIS

BARRY R. BLOOM, PH.D., *Investigator*

For many years Dr. Bloom's laboratory has been investigating basic scientific problems of particular relevance to the Third World. Tuberculosis is the major infectious disease in the world today, resulting in 3 million deaths and 8 million new cases annually. Leprosy afflicts 10–12 million people worldwide and produces deformity in 30%. Both diseases are caused by mycobacteria. The laboratory is investigating the immune responses to these organisms and the molecular basis of their pathogenesis.

Immunologic Unresponsiveness in Leprosy

One unique aspect of leprosy is that it is not a single clinical entity, but rather forms a clinical spectrum. That spectrum correlates strongly with the degree of cellular immunity of the patients. In the tuberculoid form, the lesions, which may be few, can be healed by the immune response, though often at the price of damage to nerves. At the other end of the spectrum in the lepromatous form there is an almost total absence of cell-mediated immunity to antigens of *Mycobacterium leprae*, and the bacilli ineluctably multiply within macrophages and disseminate in prodigious numbers. What is the mechanism of the specific unresponsiveness to antigens of *M. leprae* in lepromatous patients? An understanding of this immunologically fascinating phenomenon has relevance to the production of immunological tolerance, or unresponsiveness, to organ grafts, cancers, autoimmune reactions, and other infectious diseases.

Dr. Bloom's laboratory has found that T lymphocytes from lepromatous, but not tuberculoid, patients can suppress the proliferation of antigen-reactive T cells—i.e., cells that produce protective molecules such as interferon-γ (IFN-γ) and interleukin-2 (IL-2). These suppressor cells have been identified by a specific surface marker, CD8, and by a unique mode of antigen recognition. Their ability to recognize antigen molecules is controlled by a specific component of the major histocompatibility system known as HLA-DQ, whereas antigen-responding cells that produce protective responses recognize antigen in the context of another set of histocompatibility antigens, HLA-DR.

Of particular interest, Dr. Bloom's group has found that suppressor cells produce a specific pattern of lymphokines, including IL-4 and IL-5, but not IFN-γ. These cells and lymphokine patterns are found in skin lesions of patients with lepromatous leprosy. In contrast, protective T cells produce IFN-γ and IL-2, but not IL-4 and IL-5. These results point to the fact that there are different functional subsets of T cells in humans that can best be distinguished by the patterns of lymphokines they produce, and suggest that suppression and enhancement of specific immune responses can in part be attributed to those lymphokines.

Development of Recombinant BCG as a Multivaccine Vehicle

Vaccines represent the most cost-effective medical intervention in the world for preventing disease. BCG (bacillus Calmette-Guérin) vaccine, given to immunize against tuberculosis in most parts of the world, could serve as a vector for administering recombinant foreign antigens. Among advantages are its widespread use, having been given to 2.5 billion people since 1948; the very low incidence of serious side effects; the fact that it is one of the two vaccines that can be given at birth or any time thereafter; its induction of long-term sensitization (5–50 years, with a single shot); its potency as an adjuvant for animal and human use; and its extraordinarily low cost.

Over the past several years, Dr. Bloom's laboratory, in collaboration with Dr. William R. Jacobs, Jr. (HHMI, Albert Einstein College of Medicine), has developed genetic tools for introducing and expressing immunological foreign antigens in mycobacteria, including BCG vaccine strains. Because BCG takes 3–4 weeks to produce colonies, the strategy has been to develop shuttle vectors in which foreign genes are introduced and manipulated in *Escherichia coli* and then transferred to the mycobacteria.

Initial studies were carried out using temperate phages, for which in the past year the chromosomal and phage attachment sites have been defined that allow site-specific integration of single gene copies into the chromosome of BCG. In addition, a shuttle plasmid vector system has been developed that produces 10–15 gene copies per BCG cell. For both systems, in collaboration with Drs. Charles K. Stover and Vidal De la Cruz (MedImmune, Inc.), vectors have been created that utilize major heat-shock promoters, HSP60 and HSP70, to facilitate high-level expression of the introduced foreign antigens upon infection of culture cells or animals.

For the initial studies, β-galactosidase was used as a marker antigen, and later GP120 of human immunodeficiency virus (HIV) and the C fragment of tetanus toxin were tested as recombinant antigens. The genes were expressed at varying levels in heat-shocked BCG *in vitro*. The recombinant BCGs were then used to immunize mice. The results of the first such experiments have been encouraging. All three types of potentially protective immune responses—production of humoral antibodies, induction of T cell–derived lymphokines including IFN-γ, and generation of cytotoxic T lymphocytes—were produced in immunized mice. As few as 100 bacilli were capable of immunizing, and immune responses from a single inoculation persisted for more than 20 weeks.

The Killing of *Mycobacterium tuberculosis* by Activated Macrophages

Mycobacterium tuberculosis is among the microorganisms most resistant to killing. It can remain viable in air droplet nuclei for extended periods. In the body it grows primarily in mononuclear phagocytes—specialized cells for ingesting and destroying microorganisms. It is well established that reactive oxygen intermediates—superoxide anion, hydrogen peroxide, and the hydroxyl radical—represent a major cytocidal mechanism in activated macrophages. Yet experiments in Dr. Bloom's and other laboratories have indicated that virulent *M. tuberculosis* is highly refractory to killing by reactive oxygen intermediates.

In recent years a novel macrophage cytocidal mechanism appeared—namely, the production of reactive nitrogen intermediates, particularly nitric oxide (NO), within macrophages. *In vitro* macrophage-produced NO has been found to retard the growth of a number of intracellular pathogens. Mouse macrophages are very susceptible to infection and intracellular growth of *M. tuberculosis*. However, when they are activated—and in this context both interferon-γ and tumor necrosis factor-α are required—they produce NO and exert a cytocidal and cytostatic effect on *M. tuberculosis*. The NO derives from an active pathway in which the N of the guanido group of arginine is oxidized to NO. The fact that NO is responsible for killing *M. tuberculosis* was established by showing that treatment of the macrophages with the inhibitor *N*-monomethylarginine (NMMA) abrogated the killing and that the organism could be killed by pure NO in solution. The mechanisms by which the cytokines activate this pathway, the effectiveness of NO-mediated cytotoxicity *in vivo*, and the question whether human mononuclear phagocytes possess this capability remain important areas for future research.

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DEVELOPMENT OF LYMPHOCYTE SUBSETS

H. Kim Bottomly, Ph.D., *Associate Investigator*

Studies in Dr. Bottomly's laboratory focus on the development and analysis of T lymphocyte subsets. These studies include differentiation of mature $\alpha\beta$ T cells in the periphery into memory or effector T cells, as well as differentiation of subsets during intrathymic development; studies with human as well as murine T cells are included. Since these different T cell subsets perform distinct functions in host defense, characterizing their development and selective activation is crucial to understanding their behavior in infection and immunity.

Peripheral Control of CD4 T Cell Subset Activation

Studies in Dr. Bottomly's laboratory defined two populations of cloned CD4 T cells performing distinct functions and producing distinct cytokines now called Th1 and Th2. Th1 cells are specialized for macrophage activation, while Th2 cells are more effective at activating B cells to secrete antibody. Subsequent studies in Dr. Bottomly's laboratory have focused on whether such populations exist among normal antigen-specific CD4 T cells *in vivo* and what determines which subset will become activated during an immune response.

Using the immune response to a specific antigen, human collagen type IV, Dr. Bottomly has shown that a single peptide fragment of this protein activates Th1-like cells in some strains of mice and Th2-like cells in other strains. The genes determining which type of CD4 T cell becomes activated encode the major histocompatibility complex (MHC) class II glycoprotein I-A, which binds the peptide and presents it to CD4 T cell receptors. Thus, in this system at least, the determination of effector cell subtype must involve the actual ligand presented to the T cell receptor. The main focus of this line of research is to determine how a given MHC class II molecule could affect the outcome of immunization.

Three possible explanations have been considered. First, the specificity of the T cell receptor is somehow linked to the effector functions of the cell that bears it. This explanation is unlikely, since

mice transgenic for a single, rearranged T cell receptor can produce both types of effector cell, a result now confirmed in Dr. Bottomly's laboratory. Second, different antigen-presenting cells activate different subsets of CD4 T cell. This is being examined using MHC class II transgenic mice that express the class II transgene on different antigen-presenting cell subsets. The third possible explanation for the role of MHC class II molecules in determining the outcome of priming is that the density of ligand presented to the T cell receptor plays a key role.

Data obtained in Dr. Bottomly's laboratory (under a grant from the National Institute of Allergy and Infectious Diseases, National Institutes of Health) strongly support the third hypothesis: 1) MHC class II, I-A molecules that present the peptide to give dominant Th1 responses bind the peptide more strongly by several orders of magnitude than do I-A molecules that present the peptide for dominant Th2 responses; 2) adjusting peptide dose alters the functional outcome of immunization; and 3) mutant peptides with different I-A binding properties give rise to different functional outcomes. All of these data suggest that the density of ligand presented to naive CD4 T cells may dictate the functional outcome of immunization. The mechanism of this effect, and its generalization to other peptide systems, are now being explored.

Activation of CD4 T Cells Into Effector and Memory T Cells

When CD4 T cells first encounter antigen, they proliferate and subsequently differentiate into memory or effector T cells. The first question being addressed (under a grant from the National Cancer Institute, National Institutes of Health) is the nature of the memory T cell itself and its discrimination from effector T cells. Immunological memory is a critical feature of the adaptive immune response, allowing lasting protection from infectious disease by vaccination. However, it is not clear whether there exists a distinctive set of long-lived memory cells. Memory may simply be more cells of a given specificity, resulting from proliferation without differentiation following priming with antigen, or it may reflect continued activation of cells by antigen.

Naive and memory CD4 T cells have been studied using *in vitro* analysis of primary and secondary responses. Measurement of the CD4 T cell responses to non-self MHC molecules and to antigen, using T cell receptor transgenic mice, indicates that the proliferative potential of both naive and memory CD4 T

cells is identical. The differences in the magnitude and lag time of the primary and memory responses described previously for B cells can be accounted for entirely by changes in the frequency of responding cells. However, these two populations are not the same, because memory cells produce different cytokines from naive CD4 T cells. Interestingly, naive CD4 T cells make interleukin-2 (IL-2), a cytokine involved in T cell proliferation, while the memory population makes predominantly IL-4 and interferon-γ (IFN-γ), cytokines involved in the major effector functions of CD4 T cells.

These observations indicate that memory cells have differentiated so that they rapidly achieve effector function upon antigen activation, while naive cells must proliferate before effector function can be acquired. Thus immunological memory in CD4 T cells involves changes in expression of cell surface molecules and their organization (see below), increases in frequency of specific cells, and differentiation such that effector status is rapidly achieved upon antigen activation.

The second question being addressed is whether production of IL-2 and/or IL-4 is an obligatory precursor to the generation of CD4 effector or memory T cells. To answer this, mice have been generated, in collaboration with Dr. Richard Flavell (HHMI, Yale University), that carry the herpes simplex thymidine kinase gene under control of the IL-2 or IL-4 promoter sequences. When T cells are activated to make IL-2 or IL-4, they produce herpes simplex thymidine kinase, and such cells are eliminated by treating cultures with the drug ganciclovir.

Initial studies indicate that elimination of cells producing IL-2 within a primed memory cell population prevents the activation of memory CD4$^+$ T cells to secrete IL-4 and IFN-γ and interferes with the ability of memory CD4 T cells to activate B cells. These results suggest that even within the memory population, cells first produce IL-2 in response to antigen and subsequently differentiate into cells producing IL-4 and IFN-γ as well.

Role of CD45 in T Cell Signaling and T Cell Differentiation

Subsets of CD4 T cells can be distinguished by their expression of unique isoforms of the transmembrane tyrosine phosphatase CD45. There are at least six distinct CD45 isoforms that differ in the splicing of three exons that contribute to the ectodomain of the CD45 molecule. Naive CD4 T cells express mainly the unspliced isoforms of CD45, whereas memory CD4 T cells express the CD45RO

isoform, where all three variable exons have been removed. Th1 and Th2 cells also differ predictably in their expression of CD45 isoforms, suggesting a correlation between isoform expression and function/differentiation of a CD4 T cell.

Studies carried out previously in Dr. Bottomly's laboratory had shown that CD45 may influence the interaction of the T cell receptor with its CD4 co-receptor, and thus control signal transduction through the receptor. On memory CD4 T cells, CD4, CD45, and the T cell receptor appear to be associated, behaving as a single unit on the T cell surface; whereas on naive CD4 T cells, the three molecules migrate independently. These studies suggested that CD45 mediates the interplay between the T cell receptor and the CD4 co-receptor, and might influence signal transduction, perhaps mediating the differences in signal transduction between memory and naive T cells and between Th1 and Th2 cells.

To explore these issues, Dr. Bottomly has expressed a well-characterized T cell receptor, together with CD4 or CD45 or both, in a CD45-negative T cell thymoma line. Thus, using CD45 cDNAs to restore CD45 expression, cells bearing no CD45 or CD45 of one or more defined isoforms can be produced. Preliminary experiments indicate that the T cell receptor expressed alone is able to transduce signals in response to ligation with immobilized anti–T cell receptor monoclonal antibodies. CD45 transfection into cells expressing only the T cell receptor appears to have no effect on the response to T cell receptor ligation. However, when CD4 is expressed in cells having the T cell receptor without CD45, ligation of the T cell receptor no longer transduces signals measurable by cytokine release. This is surprising because this T cell receptor has been shown to be more effective at signaling in cells that also express CD4.

These prior studies were carried out in cells with mixtures of CD45 isoforms expressed. This suggests that CD45, which has been shown to play a critical role in signal transduction in T cells, acts on CD4, and biochemical data from other laboratories support this hypothesis. Studies on cells transfected with the T cell receptor, with CD4, and with individual CD45 isoforms are in progress. These should reveal whether CD45 is required for signaling in these cells, and also whether individual CD45 isoforms have distinctive effects on signal transduction. Moreover, CD45 mutants lacking tyrosine phosphatase activity or ectodomains will allow an examination of the role of different parts of this molecule in such transfectants.

Differentiation of T Cells in Murine and Human Thymus

Before T cells can be accessed by antigen in lymphoid tissues leading to adaptive immunity, they undergo a process of differentiation and selection within the thymus. The differentiative pathway appears to be preprogrammed within the cell, while selection acts on the expressed receptor. Dr. Bottomly's laboratory has examined the expression of receptors and of cytokines in human and murine thymus, using ontogenetic development to attempt to map out the sequential expression of receptor and cytokine genes.

The T cell developmental lineage splits very early into T cells expressing $\gamma\delta$ receptors and those expressing the common $\alpha\beta$ receptor found on most peripheral T cells. However, early in ontogeny, $\gamma\delta$ T cells dominate the intrathymic T cell population in mice, expressing a limited number of receptors. Using *in situ* hybridization to examine expression of $\gamma\delta$ receptor genes, Dr. Bottomly's studies showed early fetal expression of $\gamma\delta$ receptors. This pattern of early $\gamma\delta$ gene expression was not observed in human thymus, but more detailed analysis of these genes by cDNA sequencing demonstrated that the receptors found early in human ontogeny showed a sequence pattern similar to that found in the mouse. Thus there appears to be a primitive T cell receptor with very simple sequences encoded by $\gamma\delta$ genes that arises first in ontogeny, and perhaps preceded the $\alpha\beta$ receptor in phylogeny as well. Interestingly, $\gamma\delta$ T cells, whose function is not yet clear, were shown to play a significant role in pulmonary infection with influenza virus, supporting a role of these cells in epithelial defense.

Analysis of cytokine gene expression in thymus using *in situ* hybridization shows that IL-2 and IL-4 genes are expressed very early in ontogeny in the thymus, but this burst of cytokine production is rapidly extinguished during development. The expression of these cytokines and of the receptors for them is coordinated. However, the precise role of these cytokines in T cell development is not yet understood, especially since it has been reported that mice lacking IL-2 or IL-4 have normal T cell development. These studies do show that early in T cell development, IL-2 and IL-4 can be expressed coordinately in T cells, thus suggesting that the separation in production of the cytokines seen in mature effector cells using the same technique is a differentiative event in T cells.

Finally, the expression of CD45 isoforms in T cell development might be expected to play a role in selective events, since CD45 interacts with CD4,

CD8, and the T cell receptor. Dr. Bottomly's laboratory has characterized CD45 in T cell development in the thymus. These studies show that maintenance of the CD45RA high-molecular-weight isoform correlates with positive selection; and its loss, with entry of the developing T cell into a pathway leading to apoptosis. This suggests that retention of CD45 in the isoform might allow developing T cells to survive, and this is being tested using transgenic mice.

Dr. Bottomly is also Associate Professor of Immunobiology at Yale University School of Medicine and Associate Professor of Biology at Yale University.

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MOLECULAR GENETICS OF THE HLA AND CYTOKINE SYSTEMS

David D. Chaplin, M.D., Ph.D., *Associate Investigator*

Research in Dr. Chaplin's laboratory is centered on two areas: 1) analysis of the structure and function of the human major histocompatibility complex (MHC) and 2) characterization of the interleukin-1 (IL-1) family of molecules.

Structure and Function of the Human MHC

The MHC contains genes encoding the cell surface glycoproteins that present peptide antigens to α/β T lymphocytes. The human MHC, or human leukocyte-associated antigen (HLA) complex, has also been recognized to determine susceptibility to more than 200 different diseases. Because linkage disequilibrium is strong within the MHC, the molec-ular basis for most of these HLA-associated illnesses has been difficult to identify definitively. The HLA complex is recognized to span more than 4 million base pairs of DNA. Approximately 60 MHC genes have been defined, and it is currently estimated that an additional 40–100 undiscovered genes map within the complex. Understanding normal MHC immune function and the basis for many of the HLA-linked human diseases depends on defining the complete gene content of the MHC.

To obtain the nucleic acid reagents for definition of the gene content of the HLA region, Dr. Chaplin's laboratory has isolated nearly the entire human MHC as a collection of overlapping molecular

clones using yeast artificial chromosome (YAC) vectors. Studies have been initiated to apply these YAC clones to identify unrecognized genes within the HLA complex. Initial studies have been performed in a directed fashion, in which CpG islands and restriction fragments that show strong cross-species nucleotide sequence conservation have been used as possible markers of genes.

This approach has led to the identification of a novel gene located telomeric of HLA-C that is expressed exclusively in keratinocytes. The gene directs the transcription of a 2.6-kb mRNA that is expressed at high levels in normal human skin and at lower levels in transformed keratinocyte cell lines, but is absent from bone marrow–derived cells, fibroblasts, liver, kidney, muscle, lung, and brain. It predicts a protein product of 49 kDa with 28% serine residues. It shows no clear amino acid sequence homology to previously analyzed proteins. Its possible contribution to the HLA-C–linked dermatologic disorder *psoriasis vulgaris* is under investigation.

Complementary studies are under way using more systematic methods to assess gene content. In collaboration with Drs. Sherman Weissman and David Schlessinger, Dr. Chaplin's laboratory is using purified DNA from individual YACs to select clones from groups of cDNA libraries. The selected cDNAs are recovered in clonable form using PCR (polymerase chain reaction). In an initial effort using a YAC that spans the 400-kb interval from HLA-DRA to the 21-hydroxylase locus, five new genes have been identified, two of which are expressed in B lymphocytes and three of which are not. This selection methodology is powerful and rapid and is only limited by the quality of cDNA libraries available. Future extensions of this effort should permit the definition of the entire gene content of the HLA complex. (Portions of these studies using cDNA selection have been supported by a grant from the National Institutes of Health.)

Characterization of Mouse IL-1

For almost two decades it has been recognized that IL-1 is an inducible cellular protein that can potently modulate the function of most of the effector cells of the immune system as well as a broad spectrum of other cell lineages. *In vitro* studies have indicated that IL-1 has broad pro-inflammatory activities and can modulate antigen-specific immune responses. Molecular genetic and cell biological analyses have demonstrated that there are two isoforms of IL-1, designated IL-1α and IL-1β, encoded by separate, closely linked genes. Both proteins are synthesized as ~31-kDa intracellular pro-

teins and are found in culture supernatants as processed 17-kDa mature proteins. For IL-1α, both the 31-kDa pro-form and the 17-kDa mature form are bioactive. In contrast, pro–IL-1β must be processed to the 17-kDa mature form to acquire receptor-binding activity.

Previous studies in Dr. Chaplin's laboratory demonstrated that in conventional *in vitro* models of immune activation, processing and release of IL-1 from the IL-1–producing cells was inefficient, at levels consistent with release occurring due to cellular injury. Recent studies have shown that in the case of IL-1β, the nature of the cellular injury dramatically influences the outcome of IL-1 release. Injuries characterized by cellular necrosis lead to release of unprocessed, inactive pro–IL-1β, whereas cellular injuries leading to programmed cell death cause rapid, efficient conversion of pro–IL-1β to its active form. These data suggest that the primary function of IL-1 may be to signal cellular injury and to activate a systemic response.

Because the conversion of IL-1β from its inactive pro-form to the active mature form is a critical regulatory step in activation of the IL-1 response, Dr. Chaplin's laboratory has initiated studies to define the mechanisms by which this conversion is controlled. Studies using lysates of IL-1–producing cells show that pro–IL-1β is activated by a novel protease (designated the IL-1β convertase). In collaboration with Doug Cerretti, Dr. Chaplin's laboratory has isolated cDNA clones encoding the murine convertase and has determined its primary structure.

This enzyme is a novel cysteine protease that also has some structural features characteristic of serine proteases. At the mRNA level, it is constitutively expressed in a broad spectrum of cell types; however, its expression alone does not confer IL-1β–converting activity. Macrophages expressing the convertase and pro–IL-1β together show no IL-1β activation unless cellular apoptosis is induced. Preliminary data suggest that the primary translation product of the convertase is a proteolytically inactive 45-kDa proenzyme. The purified active convertase is a 22-kDa/10-kDa heterodimer.

Activation of cells to undergo apoptosis increases the convertase activity recovered in cell lysates. This suggests that activation of the apoptotic program constitutes a primary signal for conversion of the convertase from a latent cellular pro-form to its catalytically active form. Future studies will document the molecular form of the convertase in viable resting and activated cells and in injured cells undergoing necrosis or apoptosis. (Initial studies of

the IL-1 convertase were supported by funds from the Monsanto Company.)

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DEVELOPMENT OF THE IMMUNE SYSTEM

Max D. Cooper, M.D., *Investigator*

Developmental biology of the immune system is the theme of Dr. Cooper's laboratory, in which lymphoid differentiation of hematopoietic stem cells is explored. The goal is to define the abnormalities in T and B cell differentiation that occur in human immunodeficiency diseases and lymphoid malignancies.

B Cell Recognition as a Function of Differentiation

Normally we produce $\sim 10^{10}$ B cells each day in our bone marrow. This differentiation process allows the continual renewal of the B cell repertoire throughout life. The process begins when hematopoietic stem cell progeny are triggered by contact with neighboring stromal cells and their soluble products to become lymphoid progenitors. Cells

thus influenced to differentiate along this lymphoid pathway express the transmembrane CD19 molecule, and their progression is marked by the rearrangement and expression of immunoglobulin (Ig) heavy-chain (HC) and light-chain (LC) genes.

VDJ_H gene rearrangements usually occur first to allow μHC expression, and VJ_L rearrangements then occur to allow LC expression. Progenitor B cells thus lack both HC and LC, while daughter precursor B cells produce μ chains that are largely retained in the endoplasmic reticulum by a protein called BiP. Following LC gene rearrangement and expression, pre–B cells are converted into B cells as their κ or λ LC genes displace BiP to allow cell surface expression of IgM antigen receptors.

The discovery of surrogate (ψ) LC genes that do not require rearrangement to be expressed in pro–B

cells has dramatically altered this view of B cell development. Current hypothetical models suggest that ψLC proteins, encoded by λ5/14.1 and Vpre-B genes, may pair with either ψHC or μHC to form receptors that signal the progressive differentiation of pro–B, pre–B, and immature B cells. However, when monoclonal antibodies were prepared and used to examine ψLC protein expression during normal and abnormal B cell differentiation, the expression pattern was found to be different from that anticipated by these models. While ψLCs are produced during several developmental stages, their cell surface expression is restricted to a relatively late stage in normal pre–B cell differentiation.

Crosslinkage of the ψLC/μHC receptors on pre–B cells leads to receptor down-modulation and transient increase in intracellular calcium levels. However, pre–B cell growth and B cell differentiation is neither enhanced nor inhibited by anti-ψLC antibody treatment. The results indicate that ψLC/μHC receptors are not involved in negative selection of the B cell repertoire, nor are they informative about postulated positive roles in pre–B cell survival and B cell differentiation.

IgM molecules cannot reach the B cell surface by themselves, and even if they could, their short intracytoplasmic tails are inadequate for signal transduction. Ig receptors require associated transmembrane molecules both to reach the cell surface and to transduce signals after crosslinkage by antigens. Two covalently linked transmembrane molecules, encoded by the *mb-1* and *B29* genes, have been defined as integral components of the antigen receptor units on B cells. Dr. Cooper's laboratory produced monoclonal antibodies against an exposed extracellular epitope on the *B29*-encoded β chain. Cytoplasmic expression of β chains begins in pro–B cells, while surface expression occurs concurrently with expression of Ig receptors of all isotypes. Immunochemical analysis reveals molecular heterogeneity of the Ig-associated molecules that varies as a function of differentiation stage and Ig isotype.

The data support the hypothesis that biochemical heterogeneity of the surface Ig-associated molecules may contribute to the variable effects of antigen receptor crosslinkage on B cells of different maturational stages. The anti–β-chain antibodies may prove therapeutically useful as universal B cell suppressants, since they can down-modulate the antigen receptors on all B cells.

Defective B Cell Differentiation

Interruptions in key events occurring in this differentiation pathway may result in antibody defi-ciencies. X-linked agammaglobulinemia (XLA), the prototypic antibody deficiency, is characterized by recurrent bacterial infections due to a severe deficit in B cells and their mature antibody-producing progeny. The gene responsible for this inherent B cell defect is located in the Xq21.2-22 region. While all stages in B cell differentiation can be found in XLA bone marrow, the bottleneck appears to involve the pre–B cells, relatively few of which enter the cell cycle. As clonal expansion normally is prominent in this differentiation compartment, identification of the XLA gene may be the key to understanding the molecular basis for this growth spurt.

IgA deficiency (IgA-D) and common variable immunodeficiency (CVID), long considered to be unrelated and very heterogenous disorders, instead may represent polar ends of a continuous clinical spectrum. An arrest in differentiation of immature B cells is seen in both IgA-D and CVID, the chief distinction being in the Ig isotypes involved in the failure to undergo plasma cell maturation. Genetic predisposition is evidenced by variation in incidence of IgA-D from 1/500 to 1/18,500 in Caucasians and Asians, respectively, and by families in which several members with either CVID or IgA-D are seen in successive generations. Location of the underlying susceptibility gene(s) in the major histocompatibility complex (MHC) region of chromosome 6 is suggested by the frequent occurrence of certain MHC haplotypes in both CVID and IgA-D patients, and their rarity in populations in which these immunodeficiencies are uncommon.

While the highly polymorphic MHC genes tend to be inherited as a block, making precise mapping of the postulated susceptibility gene difficult, an MHC class III subregion that normally contains the C4A gene is the most frequently conserved portion of the disease-associated haplotypes. The C4A gene is often deleted in these haplotypes. It is known that the C4A complement protein preferentially binds to antigen-antibody complexes and indirectly enhances antigen triggering of B cells in antibody responses featuring isotype switching. Current efforts in Dr. Cooper's laboratory and that of Dr. John Volanakis focus on structural and functional evaluation of the C4A alleles in CVID and IgA-D patients. (The project described above was supported by a grant from the National Institute of Allergy and Infectious Diseases, National Institutes of Health.)

Dr. Cooper is also Professor of Medicine, Pediatrics, and Microbiology at the University of Alabama at Birmingham.

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MECHANISMS OF ANTIGEN PROCESSING

Peter Cresswell, Ph.D., *Investigator*

The function of membrane glycoproteins encoded by the major histocompatibility complex (MHC) is to bind peptides derived from self and foreign proteins and to present them on the cell surface for screening by T lymphocytes. The primary interests of Dr. Cresswell's laboratory are in the molecular mechanisms involved in the intracellular generation and binding of peptides and the role of peptide association in the transport and expression of MHC molecules. Much of the past year's work has centered on analysis of mutant cell lines that are defective in the peptide-loading process.

Function of MHC Class I Molecules

Two genes have been identified in the MHC that are required for the formation of complexes of class I molecules with peptides in the endoplasmic reticulum (ER). These are now referred to as *TAP.1* and *TAP.2*, and both encode multi-membrane-spanning proteins that are members of the ATP-binding cassette (ABC) family of membrane transporters. Dr. Cresswell and his colleagues have shown that a cell line (T2) lacking TAP.1 and TAP.2 proteins is impaired in its ability to transport HLA (human leukocyte-associated antigen) class I–β_2-microglobulin (β_2m) dimers out of the ER, with the single exception to date of the product of the *HLA-A2* allele. HLA class I molecules other than HLA-A2 fail to bind peptides when expressed in T2. HLA-A2, however, binds peptides derived from a limited number of signal sequences when it is expressed in T2, explaining its transport and cell surface expression. Thus, at least for the *HLA-A2* allele, signal sequences may represent an alternative source of antigenic peptides for the generation of class I–restricted T cell epitopes. (This work was supported by a grant from the National Institutes of Health.)

Transport of mouse (H-2) class I glycoproteins in

T2 is much less affected than that of HLA molecules. In this case, transport occurs despite the lack of associated peptides. The precise cellular mechanism responsible for the discrimination between "empty" H-2 class I–β_2m complexes and "empty" HLA class I–β_2m complexes is unclear, but may relate to differential affinity of these molecules for intracellular retention factors important in class I–restricted antigen processing.

In collaboration with Dr. Thomas Spies (Dana Farber Cancer Center), an antiserum to a synthetic peptide corresponding to the carboxyl terminus of the TAP.1 protein was used to show that the TAP.1 and TAP.2 proteins coassociate. This may reflect dimer formation and suggests that dimers of TAP.1 and TAP.2 form the structure critical for transport of cytosolic peptides into the ER. Reconstitution of mutant cell lines lacking TAP.1 or TAP.2 with the gene encoding the missing protein clearly restores the class I–restricted antigen-processing function.

Function of MHC Class II Molecules

The α and β subunits of class II MHC molecules assemble in the ER with the invariant chain, a type II transmembrane protein that is proteolytically removed from the class II–$\alpha\beta$ dimer in a post-Golgi endosomal compartment. Dr. Cresswell and his colleagues have shown that the class II invariant-chain complex is a nine-subunit structure, containing three α subunits, three β subunits, and three invariant-chain molecules. In class II–negative, invariant-chain–positive mutant cells, the invariant chain forms a trimer. In wild-type cells the invariant chain also rapidly forms trimers upon synthesis in the ER and subsequently binds α and β subunits, accumulating them to form the nine-chain structure. After transport through the Golgi, the invariant chain is degraded to release $\alpha\beta$ dimers associated with peptides.

Dr. John Newcomb, an HHMI associate in Dr. Cresswell's laboratory, has isolated and sequenced a number of the peptides associated with the HLA-DR11 molecule. They vary in length from 13 to 16 amino acids. Eight of nine peptides sequenced were from known sources, either from secretory proteins, the extracytoplasmic regions of transmembrane proteins, or heat-shock proteins.

The first two types (secretory and transmembrane protein-derived peptides) are consistent with the notion that class II molecules acquire endosomally generated peptides. Peptides derived from heat-shock proteins are more surprising. They may result from transport of these proteins into lysosomes from the cytosol, a process known to occur when cells undergo serum deprivation. No peptides were found associated with $\alpha\beta$–invariant-chain complexes, consistent with previous observations from the laboratory suggesting that one function of the invariant chain is to prevent class II molecules from binding inappropriate peptides in the early stages of transport.

Dr. Cresswell and his colleagues have shown that the T2 cell line, defective in class I–restricted antigen processing, is also defective in class II–restricted antigen processing. T2 transfectants expressing HLA-DR or I-A^k molecules assemble class II–invariant-chain complexes normally. These complexes are transported through the Golgi, the invariant chain is degraded, and $\alpha\beta$ dimers are expressed on the cell surface. However, these dimers do not contain normally processed peptides and are unable to stimulate antigen-specific class II–restricted T cells. They also lack the stability of association in SDS (sodium dodecyl sulfate) characteristic of class II–$\alpha\beta$ dimers. When peptides were isolated from HLA-DR3 molecules purified from T2 transfectants, they all proved to be derived from a small region of the invariant chain (residues 81–104). This may define the region responsible for blocking access of peptides to invariant-chain–associated class II molecules, a region that is replaced in wild-type cells by peptides generated from endocytosed proteins.

The nature of the genetic lesion in T2 responsible for the class II antigen-processing defect is under investigation. The affected gene (or genes) is clearly in the class II region of the MHC, since T2 has a large deletion in this region and the parental cell line with an intact MHC has normal antigen-processing functions. However, the precise nature of the defect remains unknown.

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MOLECULAR APPROACHES TO LYMPHOCYTE RECOGNITION AND DIFFERENTIATION

Mark M. Davis, Ph.D., *Investigator*

T lymphocytes play a number of critical roles in the immune system. Some subsets of T cells are able to kill virally infected or transformed cells directly; others seem specifically designated to mobilize other cells, particularly B cells, in the course of an immune response. Both activities can be mediated through the same recognition apparatus, the $\alpha{:}\beta$ T cell receptor (TCR) heterodimer, in close association with the CD3 polypeptides. A central feature of T cell recognition by this receptor is that antigens are often (if not always) "seen" as peptide fragments complexed with either class I or class II molecules of the major histocompatibility complex (MHC). One goal of the laboratory is to understand at a biochemical and structural level how this is accomplished topologically, with what affinity, and whether this phenomenon is distinguishable fundamentally from antibody-antigen interactions.

These pursuits have involved expressing both TCR and MHC class II heterodimers in a lipid-linked form so that they may be easily cleaved off cell surfaces and used to reconstitute T cell recognition (together with specific peptide antigens). Having tens of milligrams of soluble TCR and MHC proteins has enabled Dr. Davis and his colleagues to embark on many projects. The development of a new strategy to delineate which parts of the TCR are contacting which residues on the peptide is also providing new information on the topology and "rules of engagement" concerning T cell recognition.

Another goal is to use transgenic mice to analyze the selection of specific receptor chains and heterodimers in the thymus. This should be informative with respect to both the removal of self-reactive TCRs in the thymus (negative selection) and the enhanced maturation and export of T cells having a good "fit" with one or more of the thymic MHC molecules (positive selection). These mice are also a valuable source of physiologically normal T cells at different stages of differentiation to use in defining the requirements for activation and differentiation of these cells *in vivo*.

Deriving a Topology for TCR–mediated Recognition of Peptide-MHC Complexes

Dr. Davis and others have proposed a model (based on patterns of TCR diversity and MHC and immunoglobulin [Ig] structures) in which the V-J junctional regions of TCRs are largely or solely responsible for peptide binding, whereas germline V region–encoded loops might contact the α helices of MHC molecules. Correlative data from many laboratories indicate an important role for the V-J junctional sequences in T cell specificity, with specific residues sometimes appearing in V_α, V_β, or both in response to a specific peptide-MHC combination.

In one series of experiments, Dr. Davis and his colleagues have attempted without success to transfer peptide specificity from one TCR to another by swapping V(D)J junctional sequences and other CDR loops. As negative results in this type of experiment are difficult to interpret, the group recently developed a way of addressing the issue that has turned out to be informative. The method works by 1) introducing changes in the peptide at positions that alter T cell reactivity but not MHC binding, 2) immunizing mice that are transgenic for either α- or β-chain TCR that recognizes the original peptide (to hold half of the TCR "constant"), and then 3) analyzing the endogenous TCR usage of responding T cells. In this fashion the laboratory has elicited reciprocal changes in TCR α- and β-chain CDR3 sequences in response to changed amino acids on the peptide. This shows that there is direct TCR-peptide contact, that it is largely the province of CDR3 residues, and that both α and β chains can participate in peptide recognition.

One surprising outcome of the above studies is

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that some peptide substitutions affect V_β or V_α usage as well, suggesting that V-region structures can differ significantly from one another and limit the peptide contacts that can be made. Thus, although these results now constitute the most convincing demonstration of the original CDR3 → peptide contact model, there are clearly other aspects to the problem that require careful study. (A grant from the National Institutes of Health provided support for the project described above.)

In addition, the group has made a series of mutants that are predicted to point "up" toward the TCR on both α helices of E^k. Most of these mutants bind peptide normally, but several interfere with TCR recognition and/or superantigen reactivity. Work in progress may reveal which of these mutated residues interacts with what parts of the TCR.

The laboratory has also recently derived monoclonal antibodies specific for a particular peptide-MHC complex. Studying how these antibodies bind may give useful insights into the way B and T cell antigen receptors solve the same "problem."

Peptide-MHC Interactions

Dr. Davis and his colleagues have successfully made GPI-linked chimeras of an $\alpha\beta$ TCR and of the class II MHC molecules I-E^k. In particular, an I-E^k chimera on the surface of fibroblast cells can present peptides to most T cells (albeit at a reduced efficiency) but cannot present processed antigen. Cleaved soluble protein can present peptides efficiently and specifically to T cells when immobilized on plates, and the efficiency of antigen-MHC complex formation and stimulation of T cells is significantly greater at pH 5.0 than at neutral pH. This is consistent with the biology of class II MHC molecules as they cycle through endosomal compartments, some of which approach this pH in acidity.

The laboratory has measured the kinetic parameters of the peptide presentation effect and find that for two different peptides, the on-rate increases 50-fold at pH 5.0 (vs. 7.3). In the case of the cytochrome peptide, there is only a modest change in the off-rate. In contrast, a second, more weakly antigenic peptide has a similar increase in its on-rate (at pH 5.0) but is 100-fold weaker than the cytochrome peptide, which can efficiently displace it. This provides a model system for how competition between peptide fragments may occur in the cell and suggests that "good" peptide antigens may be strongly selected for their ability to stay bound to an MHC molecule. In addition, the generation of monoclonal antibodies specific to a cytochrome peptide-E^k complex (using soluble material as the immunogen) provides a useful reagent for determining where in the cell substructure peptides first bind to class II MHC.

Antigen-MHC and TCR Structure

Dr. Davis's group, in collaboration with Dr. Paul Driscoll (Oxford University) and Drs. Ettore Appella and Jim Omishinki (National Institutes of Health), is using two-dimensional nuclear magnetic resonance (NMR) spectroscopy to try to visualize the solution structure of a peptide in a class II MHC molecule. They have successfully prepared large quantities (20 mg) of soluble I-E^k, containing both ^{13}C alanine-labeled cytochrome peptides, and have obtained information about its conformation and major areas in contact within the MHC. They are now analyzing a fully ^{13}C- and ^{15}N-substituted peptide to get a more detailed picture of how the peptide binds to the E^k molecule. This information will also aid computer models of E^k based on class I structures and the peptide made in collaboration with Drs. Sebastian Doniach and Michael Levitt (Stanford University).

X-ray crystallographic work will continue, especially on the promising soluble TCR and peptide-MHC complex crystals obtained (with materials supplied by Dr. Davis) in Dr. Pamela Bjorkman's laboratory (HHMI, California Institute of Technology). This latter has the advantage over the NMR study in potentially providing information about the entire MHC molecule. Ultimately the goal is to cocrystallize the soluble TCR with the antigen-MHC complex.

Affinity of T Cell Recognition

Dr. Peter Parham once remarked that "the T cell receptor is the only receptor that has never been shown to have received anything." Regardless, Dr. Davis and his colleagues have been working for many years to try to analyze the kinetic parameters of TCR recognition, making use of the group's soluble molecules. Success has finally come through use of soluble peptide-MHC complexes to compete with a labeled anti-TCR antibody fragment. In the several cases studied, the group derived values of $4–6 \times 10^{-5}$ M, 1,000- to 10,000-fold weaker than for antibodies to comparable-sized protein ligands. These measurements have recently been confirmed using soluble TCR to compete with a peptide-MHC complex–specific antibody for its ligand.

These values are consistent with the "scanning" nature of T cell recognition and indicate that TCR binding to peptide-MHC ligands is so weak energetically that other, antigen-independent receptor-ligand systems must govern the initial stages of T cell contact with antigen-presenting or target cells. The best candidates for such regulators of T cell in-

teraction are adhesion molecules, such as CD28 or CD2. This would give adhesion molecule-ligand interactions a major role in "orchestrating" which T cells interact with which antigen-presenting cells. This has important implications for autoimmunity, in that normally T cells would be expected to "focus" on appropriate antigen-presenting cells, such as B cells or macrophages, and would be hindered in surveying most other cells or tissues (which would lack the appropriate ligand expression).

Requirements for T Cell Activation and Inactivation

How T cells become activated and under what circumstances they are inactivated in the periphery are central issues in their biology. The laboratory has developed a procedure whereby primary CD4$^+$ T cells are purified from TCR-$\alpha\beta$ transgenic lymph nodes and stimulated with antigen-MHC complexes, either coated on plates or on cells, alone or in combination with various putative co-stimulator molecules (or antibodies directed at such molecules). In this way the minimum requirements for T cell activation may be determined and possible scenarios for inactivation (anergy) investigated.

In preliminary results, stimulation of either primary or secondary T cells through the TCR alone does not appear to result in anergy. Stimulation through the protein kinase C (PKC) pathway is required for interleukin-2 (IL-2) release in the former but not the latter cells.

This last point links T cell memory with models of neuronal memory, where PKC activation is also associated with the establishment of a learned response. By using "degenerate" PCR (polymerase chain reaction) methodology, the laboratory has identified a novel PKC isoform (PKC-T), which is expressed specifically in T cells, but not B or other circulating cells. This molecule may be an important missing link in the control of T cell stimulation in ontogeny and in the periphery. (The projects in this section have been funded through a grant from the National Institutes of Health.)

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GENETICS, STRUCTURE, AND FUNCTION OF HISTOCOMPATIBILITY ANTIGENS

KIRSTEN FISCHER LINDAHL, PH.D., *Investigator*

Dr. Fischer Lindahl's laboratory studies the mouse major histocompatibility complex (MHC) and the molecules it encodes. Projects have focused on antigen presentation by two medial MHC class I molecules, H-2M3 and Qa-1; on evolution and intraspecies divergence of β_2-microglobulin (β2m), a component of all MHC class I antigens; and on mapping, cloning, and characterizing genes in the mouse MHC's *M* region and *S-D* interval.

H-2M3

H-2M3 was defined as the MHC class I molecule that presents the maternally transmitted antigen of mice, which is a mitochondrially encoded, *N*-formylated peptide. An amino-terminal formyl group is essential for binding of peptides to M3. Mitochondrial and prokaryotic proteins share this feature, which distinguishes them from proteins synthesized on cytosolic ribosomes, and it was therefore reasonable to assume that M3 might also present bacterial peptides and play a role in the immune response against infection. This has now been confirmed through a collaboration with the laboratory of Dr. Michael Bevan (HHMI, University of Washington).

Dr. Eric Pamer (University of Washington) has

studied the specificity of cytotoxic T lymphocytes (CTLs) derived from mice infected with *Listeria monocytogenes*. He isolated one clone (CD8+ with αβ receptors) that reacted with infected cells of several *H-2* haplotypes. This clone detects a peptide, Fr38, isolated by high-pressure liquid chromatography as fraction 38, which is produced by the bacteria rather than the infected cells and has a blocked amino terminus. The clone reacts with Fr38-pulsed target cells from many strains but not from Mta⁻ strains, including the B10.CAS2 fibroblast line, and not with β2m-deficient cell lines. Because the clone recognizes Fr38-pulsed B10.CAS2 cells transfected with a cosmid that carries *H-2M3ᵈ* as its only class I gene, it was concluded that H-2M3 presents the *Listeria* peptide.

The *Listeria*-specific T cell clone failed to recognize Fr38 on cells from a number of wild-derived strains that can present the Mta peptide to bulk-cultured CTLs. These results suggest that the M3 molecules from the wild and laboratory mice differ by a few amino acids, enough to abolish recognition by a single receptor species but not by a wider repertoire of receptors. These new alleles, which are being characterized by sequencing, show that *H-2M3* is more polymorphic than was originally suspected. In this respect too, the gene is a neoclassical class I gene. Like classical class I antigens, *H-2M3* is expressed from before day 8 of embryonic life, and its expression is inducible with interferon-γ.

H-2M2 and *M3* are the most distal class I genes in the mouse MHC. Dr. Elsy Jones has isolated one yeast artificial chromosome (YAC) with *M2* and two with *M3*, all in the range of 250 kb. Surprisingly, these clones contain no other MHC class I genes (which otherwise tend to be clustered less than 20 kb apart in the central *D, Q,* and *T* regions of the mouse MHC). By pulsed-field electrophoresis, Dr. Jones has also succeeded in linking the *M1-M7-M8* and the *M4-M5-M6* class I gene clusters from the proximal part of the *M* region on an 870-kb *Not*I and a 245-kb *Sfi*I fragment. The spatial isolation of the expressed *M2* and *M3* genes may contribute to their conservation, protecting them from loss by unequal recombination between tandemly aligned, homologous genes.

RMA-S Cells and Qa-1

RMA-S mutant cells produce empty, unstable class I molecules lacking peptides, and they cannot present antigens derived from endogenously synthesized proteins; surface display of stable, complete class I molecules can be restored by addition of appropriate synthetic peptides. Evan Hermel, a graduate student in the laboratory, showed that the RMA-S

mutation also affects display of the medial class I antigens Mta and Qa-1. In a collaboration with Dr. John Monaco (Medical College of Virginia, Richmond), expression of both medial and major class I antigens was restored when the cells were transfected with a functional *Tap-2* gene, an MHC gene that encodes a member of the ABC superfamily of transporter proteins.

In a collaboration with Drs. Carla Aldrich and James Forman (University of Texas Southwestern Medical School at Dallas), RMA-S cells were used to dissect the peptide specificity of Qa-1ᵇ–specific CTL clones. Qa-1 was the second medial class I antigen to be described; the Qa-1ᵇ antigen of RMA-S cells is encoded by the *T23* gene. Up to half the clones generated from a secondary *in vitro* response lyse RMA-S cells at least as well as RMA. These clones may recognize empty Qa-1 molecules or, more likely, Qa-1 molecules with a peptide, such as a signal peptide, that does not require a functional TAP transporter to enter the endoplasmic reticulum. The rest of the clones require a functional *Tap-2* gene and hence recognize peptides that are not otherwise transported into the endoplasmic reticulum.

Among such clones, some fail to recognize target cells that are homozygous for the *H-2D* region of the *k* haplotype; *Qdm*, a gene in this region, affects the ability of target cells to be recognized by anti–Qa-1 CTL. The simplest explanation for this observation is that Qa-1 can present a peptide derived from any H-2D molecule except H-2Dᵏ, and this peptide requires a functional TAP transporter too.

A further distinction can be made among *Tap-2*–dependent, *Qdm*-independent clones. Some recognize RMA-S target cells that have been treated with oligomycin, an inhibitor of mitochondrial ATPase, which also increases lysis by Mta-specific CTLs, or with ionomycin, a calcium ionophore. The increase in intracellular calcium caused by the drugs may stimulate proteolysis, and the *Tap-2* defect may be overcome by the increased concentration of peptides. One clone kills RMA-S treated with oligomycin but not ionomycin.

Interspecies Divergence of *B2m*

Mice are the only mammals in which allelic forms of β2m have been documented. Because of its intimate interaction with the peptide-binding α1 and α2 domains of MHC class I molecules, allelic differences of β2m can subtly affect the antigenic structure of MHC class I molecules and their ability to bind particular peptides. Evan Hermel has sequenced the second exon of the *B2m* gene from several species of wild mice. This exon encodes 92 of the 99 amino acids in mature β2m. Whereas four

alleles of *Mus musculus* origin differed by only one or two coding and no silent nucleotide changes from the *B2m^a* allele of inbred mice, it was a surprise that the *B2m* gene from *Mus spretus* differed by 14 nonsynonymous changes and only one silent change. This contrasts with the modest divergence seen in other members of the immunoglobulin superfamily.

By comparison, the *B2m* genes of rats and mice differ by a similar number (14) of amino acid changes but a total of 52 nucleotide changes. In collaboration with Dr. Jin-Xiong She (University of Florida, Gainesville), this observation has been confirmed in five independent isolates of *Mus spretus*, which do not differ from each other, and extended to *Mus spretoides, cervicolor,* and *spicilegus*. The paucity of silent relative to coding changes suggests that diversifying selection accompanies speciation. The changes in the β2m molecule are concentrated on the face away from the MHC class I heavy chain, while the face that interacts with the α3 domain is conserved.

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TOLERANCE, AUTOIMMUNITY, AND THE MAJOR HISTOCOMPATIBILITY COMPLEX

RICHARD A. FLAVELL, PH.D., *Investigator*

Tolerance, Inflammation, and Autoimmunity

The relationship between immune tolerance and maintenance of an effective T cell repertoire is a balance that, when upset, can result in self-reactive lymphocytes and autoimmunity. What causes this breakdown of tolerance in autoimmunity is not understood. Although central tolerance in the thymus eliminates most self-reactive T cells, extrathymic

mechanisms also exist. In autoimmune diseases, tolerance to peripheral antigens is lost and immune destruction of a specific cell type occurs, e.g., the islets of Langerhans in insulin-dependent diabetes mellitus (IDDM).

Studies of tolerance have been facilitated by transgenic mice expressing a tissue-specific antigen and by utilizing, as a source of T cells, transgenic mice

that express one rearranged T cell receptor (TCR) on all T cells. By crossing TCR transgenic mice with mice expressing the antigen, tolerance can be directly studied. Dr. Flavell's laboratory described a system with simian virus 40 (SV40) T antigen (SV-T) as tissue-specific antigen and an $\alpha\beta$ TCR from a cytotoxic T lymphocyte (CTL) that recognizes T antigen. When antigen was expressed postnatally, spontaneous autoimmunity resulted in double (TCR plus antigen) transgenic mice. A working hypothesis has been that the inflammatory effects of SV-T (e.g., tissue hyperplasia, necrosis) initiate this autoimmunity, causing presentation of SV-T to nontolerant T cells.

To dissect the role of inflammation in autoimmunity, Dr. Flavell's laboratory created a chronic tissue-specific inflammatory state by directing the synthesis of the inflammatory cytokines tumor necrosis factor-α (TNF-α) and TNF-β to the islets of Langerhans in transgenic mice. Production of either cytokine is sufficient to cause a massive lymphocytic inflammatory infiltrate. Inflammation does not lead to autoimmunity, since these mice never develop diabetes. An inflammatory response seems therefore not to be a sufficient condition for autoimmunity. (A grant from the National Institutes of Health provided partial support for the project described above.)

A likely explanation for the absence of tissue destruction in these mice is the failure of the lymphocytes to become activated. Two transgenic models have been developed where T cells can be activated locally. In the first, interleukin-2 (IL-2) was directed to the islets of Langerhans to test whether it could cause autoimmunity, e.g., by the activation of "anergic" T cells that were previously tolerant. IL-2, however, has several other known abilities to activate lymphocytes. Transgenic mice expressing IL-2 in the islets develop a strong infiltrate predominantly of T cells and become diabetic at approximately 4–5 months of age. Current efforts are directed toward determining whether this diabetes is autoimmune.

A second test of the role of T cell activation in autoimmunity involves co-stimulatory molecules. The two-signal model of T cell activation states that naive T cells are activated only when stimulated through the antigen receptor and simultaneously through a second receptor that delivers a co-stimulatory signal. T cells receiving stimulation only through the antigen-specific receptor are believed to be inactivated or anergized. A favored molecule for the co-stimulatory receptor on T cells is CD28; its ligand is the molecule B7, found on pro-

fessional antigen-presenting cells (APCs). If this hypothesis is correct, then tolerance would be mediated by stimulation of T cells by antigen presented on tissue cells that lack B7, such as islets of Langerhans. Conversely, expression of co-stimulatory ligand B7 on the islets in conjunction with MHC should lead to T cell activation. In this way the islet would become a quasi APC.

Transgenic mice expressing the human (h) B7 have been generated, and also transgenic mice coexpressing both hB7 and the MHC class II molecule I-E. Islets transgenic for hB7 possess APC function *in vitro* and are capable of activating naive CD8 T cells and thus of providing co-stimulatory function. Despite this, transgenic mice expressing hB7 alone do not become autoimmune. However, mice expressing hB7 and I-E on the islets acquire a lymphocytic infiltrate. Current efforts will test whether this is an autoimmune response. If so, the two-signal hypothesis will be strongly supported.

Protective Role of MHC in Autoimmune Diabetes

The murine I-E molecule protects against diabetes in nonobese diabetic (NOD) mice. Some time ago Dr. Flavell's laboratory, in collaboration with Dr. Ralph Brinster (University of Pennsylvania) and Dr. Richard Palmiter (HHMI, University of Washington), generated transgenic mice in which the expression of the I-E molecule was directed to specific tissues that are a subset of the I-E$^+$ tissues in normal I-E$^+$ mice. These mice therefore permit the determination of the cell type that mediates this protective effect. Accordingly, Dr. Flavell's group, in collaboration with Dr. Pia Reich and Dr. Charles Janeway (HHMI, Yale University) crossed these transgenic mice to the NOD background and showed that only transgenic mice expressing I-E on peripheral APCs were protected from IDDM. These results suggest that the protective effect is mediated by antigen presentation, either by the induction of tolerance or by the preferential activation of "protective" T cells. Positive selection of T cells by I-E does not play a major role, since mice lacking I-E on the thymic cortex were still protected against diabetes. (A grant from the National Institutes of Health provided partial support for the project described above.)

Regulation and Expression of the MHC

Dr. Flavell's laboratory has isolated mutant HeLa cell lines defective in the transcriptional activation of MHC genes by interferon-γ (IFN-γ). The first group of mutants were defective in the transcriptional activation of both IFN-γ– and IFN-α–

regulated genes, showing overlap between IFN-γ and IFN-α gene regulation. These mutants, which fall into one complementation group, are defective in an early event during activation of the transcription factors mediating IFN-α and IFN-γ effects. The group of Dr. James Darnell (Rockefeller University) has recently shown that a transcription factor that mediates IFN-α activation (ISGF3-α) also contains a subunit that is activated by IFN-γ (GAF). In collaboration with Dr. Darnell, Dr. Flavell's group has shown that these mutants fail to phosphorylate both the γ and α subunits, upon stimulation of the receptors. These polypeptides seem nonetheless to be present within the cells, pinpointing the likely defect at an early stage of phosphorylation of these proteins prior to nuclear translocation. The mutants also fail to translocate these factors to the nucleus.

The second major class of mutants cannot transactivate class II MHC and the gene for the class II–associated polypeptide in the invariant chain. The first three mutants analyzed in this group also consist of a single complementation group. cDNA expression libraries are being used to complement these defective mutants in order to clone the gene responsible for the defect.

The invariant chain is believed to play a role in the association of antigenic peptides with MHC. It probably targets MHC class II to endosomes where antigen is bound and the invariant chain dissociated. To elucidate the function of invariant chain *in vivo*, Dr. Flavell's laboratory has generated "knock-out" mice defective in their endogenous invariant chain genes, by the use of homologous recombination. These mice are under analysis to identify their immune defects and elucidate further the role of the invariant chain.

Lineage Relationships and Biologic Role of CD4 and CD8 T Cell Subsets

Although the development of T cells in the thymus is becoming clear, T cell differentiation in the periphery is poorly understood. CD4 T cells fall into two classes expressing different cytokines. TH1 cells produce IFN-γ, IL-2, and TNFs, whereas TH2 cells produce IL-4, IL-5, and IL-6. The lineage relationships between these cells and precursors that exit the thymus are not understood. To elucidate this and to obtain a better understanding of their functional role, Dr. Flavell's laboratory, in collaboration with Dr. Kim Bottomly (HHMI, Yale University), has developed a transgenic model whereby such cells can be specifically ablated. The IL-2 and IL-4 promoters have been used to direct the synthesis of HSV-TK (herpes simplex virus thymidine kinase) to the subsets that produce these cytokines.

Upon activation of these genes, the cells proliferate and can be killed by ganciclovir; this approach was developed originally for other systems by Dr. Ronald Evans (HHMI, Salk Institute for Biological Studies). Transgenic mice utilizing the IL-2 promoter produce T cells that are readily susceptible to killing by ganciclovir *in vitro*. The validation of this system in a series of standardization experiments has shown that it works and is capable of addressing the questions posed. Current activities, therefore, are directed at elucidating the precise relationship of IL-2–, IL-4–, and IFN-γ–producing CD4 T cell subsets.

Dr. Flavell's laboratory has used these mice to address the differentiation of CD8 T cells. It is known that some CD8 T cells produce IL-2 (CD8 helper cells), whereas others develop effector function and become CTLs. The relationship between CD8 helper cells and CTLs is not clear. This laboratory has utilized T cells from TCR transgenic mice, doubly transgenic for the IL-2–TK (TK, thymidine kinase) construct, to answer this question. Growth *in vitro* of such cells, in the presence of antigen and ganciclovir, eliminates their proliferative response and the production of CTLs. Since the CTLs are not restored by the addition of IL-2, this experiment suggests that the so-called CD8 helper cells are precursors of the CTLs or even the same cell.

Protective Immunity in Lyme Disease

Lyme disease is a debilitating inflammatory disease caused by the spirochete *Borrelia burgdorferi* when transmitted by tick bite to humans and animals. Originally considered to be a focal disease in small areas of the United States, Lyme disease is now realized to be worldwide and has been detected on all continents except Antarctica. Protective immunity was shown by this research group, in collaboration with the groups of Drs. Fred Kantor and Stephen Barthold (both of Yale University), to be mediated by antibody to outer surface protein A and B (OspA and OspB), but not the flagellar protein. Vaccination with either OspA or OspB fully protected mice against Lyme disease, whether transmitted by injection or tick bite. This experimental vaccine is currently being evaluated for safety prior to human clinical trials. For the vaccine to be clinically effective, humans must make neutralizing antibodies. Dr. Flavell's group has now shown that passive transfer of antibodies, from humans that are seropositive for OspA and OspB, protects mice from infection with *Borrelia*. This suggests that humans make protective antibodies, which enhances the prospects for a human vaccine.

Last year Dr. Flavell's group reported that vacci-

nated mice were protected against infection by tick-borne *Borrelia*, and further that spirochetes were eliminated from infected ticks. This has made possible an epidemiologic study of the efficacy of protective immunity, since ticks can be collected in the wild and their ability to infect laboratory mice assayed. Although the results from any individual tick cannot be directly evaluated, the analysis of sufficiently large numbers of ticks will provide a statistical sample that will test the efficacy of the vaccine in wild ticks. The first such study has been performed using ticks from Nantucket. Vaccination with OspA or OspB was effective against infection with wild ticks; and again the spirochetes were eliminated from infected ticks. These studies are now being extended to ticks from other locations. It should therefore be possible to predict the breadth of protection that can be afforded by OspA and OspB, prior to human clinical studies.

Research in the past year has also focused on mechanisms whereby the spirochete evades protective immunity. Spirochetes have been identified in which one mechanism of evasion appears to be truncation of the OspB polypeptide. As Dr. Flavell's group showed previously for OspA, neutralizing determinants on OspB are found near the carboxyl terminus. A mutant form of *B. burgdorferi* strain N40 has a truncated OspB that permits escape from immune surveillance in vaccinated mice, in contrast to wild-type *Borrelia*, which lacks this truncation. Furthermore, live spirochetes recovered from vaccinated mice show alterations in outer surface proteins. These are now being characterized. (Grants from the National Institutes of Health, the Centers for Disease Control, and the Mathers Foundation provided partial support for the project described above.)

Dr. Flavell is also Professor of Immunobiology at Yale University School of Medicine.

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REGULATION OF NF-κB AND IκB DURING B CELL DEVELOPMENT

SANKAR GHOSH, PH.D., *Assistant Investigator*

The process of differentiation and development of B cells from pluripotent stem cells serves as a useful experimental model to study various aspects of mammalian ontogeny. This process is characterized by regulated expression of different genes at different stages. Such developmentally regulated genes include the immunoglobulin heavy and light chains, λ5, VpreB, *B29*, and *mb-1*. The major interest in Dr. Ghosh's laboratory is to understand how the expression of one of these genes, the immunoglobulin κ light-chain gene, is regulated.

The expression of the κ gene depends on the activity of two enhancers, one located in the intron between J$_\kappa$ and C$_\kappa$ exons and the other ~9 kb downstream of the C$_\kappa$ gene. The activity profiles of the two enhancers overlap to a great degree during B cell development, although plasma cell lines in which the intronic enhancer is inactive are still able to express large amounts of κ mRNA, indicating the importance of the 3' enhancer in later stages of B cell development. However, it is the intronic enhancer whose activity correlates exactly with the transition from a pre–B to a mature B cell.

The developmental and tissue-specific activity of this enhancer depends on a transcription factor, NF-κB, which belongs to the *rel* family of proteins. Chromosomal translocations involving members of this family have been linked to the development of human B cell lymphomas. In addition to its central role in the expression of the immunoglobulin κ gene, NF-κB is also an important element in the inducible expression of a wide variety of cellular genes and in the replication of various viruses, including human immunodeficiency virus (HIV). An understanding of the molecular mechanisms that regulate the function of NF-κB should therefore help to illuminate fundamental principles in oncogenesis, as well as normal development of B cells.

Toward that goal, Dr. Ghosh's laboratory is focusing on IκB, an inhibitory protein that regulates the activity of NF-κB. In pre–B cells the ubiquitous NF-κB is present in an inactive form, being bound to IκB. However, in mature B and plasma cells, NF-κB is a constitutively active, nuclear protein. Therefore the change of NF-κB from an inducible cytosolic protein to an active, nuclear protein is linked intimately to the progression of B cell development. The recent cloning of the genes encoding the subunits of NF-κB (p50 and p65) and IκB finally allows a detailed analysis of the regulation of NF-κB activity in B cells.

An important question that remains to be answered is, What causes the constitutive activation of NF-κB in mature B cells? Using Northern and Western analyses, Dr. Ghosh's laboratory has found that IκB mRNA and protein continue to be made in mature B and plasma cells, yet the protein in these cells cannot bind to NF-κB. One possible explanation is that IκB in these cells is post-translationally modified and inactivated. Alternatively, the IκB in mature B and plasma cells is sequestered by binding to a new protein whose synthesis is regulated during development. Experiments are now in progress to determine if either of these or yet some other mechanism is responsible for regulating IκB activity in later stages of B cell development.

IκB, like NF-κB, is also a member of a family of proteins that share a common structural motif known as ankyrin repeats. At present three different IκBs have been cloned—IκB-α, IκB-γ, and the proto-oncogene *bcl-3*; another form, IκB-β, is yet to be cloned. The major form, IκB-α, is not regulated through transcription in B cells (see above), but the expression of IκB-γ (an alternatively spliced version of the p50 NF-κB precursor) is regulated at the level of mRNA. Both pro– and pre–B cells have significant amounts of the IκB-γ message, while mature B and plasma cells have almost none.

Unlike IκB-α, which interacts specifically with the p50:p65 NF-κB heterodimer, IκB-γ appears to target the p50 dimer. It is not clear what role the p50 dimer, and by extension IκB-γ, play during B cell differentiation. Because IκB-γ levels decrease upon differentiation, it is likely that IκB-γ is important to maintain an undifferentiated state during earlier stages of development. Therefore inhibition of IκB-γ expression in pre–B cells may lead to differen-

tiation and may result in the rearrangement and expression of the κ locus. Alternatively, inappropriate expression of the IκB-γ message in mature B or plasma cells may affect the expression of genes specific for the later stages of B cell development.

To test these hypotheses, Dr. Ghosh and his colleagues will do experiments to alter the level of IκB-γ message in cells, using either antisense RNA for reducing levels of expression or strong constitutive promoter/enhancers for elevating levels of expression. These studies will also be extended to animals, using transgenic techniques. Transgenic mice will be made using promoter/enhancer combinations that are active throughout B cell development, e.g., the immunoglobulin heavy-chain promoter/enhancer, to drive the expression of IκB-γ. Such inappropriate expression may lead to a block in the differentiation of B cells at a stage where, under normal circumstances, IκB-γ expression would be shut off, and such blockage may disrupt the normal balance between differentiation and proliferation. As a consequence one may observe the generation of B cell lymphomas similar to that observed in the case of dysregulated expression of another putative IκB, bcl-3. This is a putative oncogene whose elevated expression due to a chromosomal translocation leads to development of B cell lymphomas. Although a recent report indicates that bcl-3 in vitro preferentially interacts with the p50 dimer, it is still unclear if p50 is the physiological target in vivo. Therefore it appears that inappropriate inhibition of the function of certain rel transcription factors (the

target of bcl-3) can cause transformation of pre–B cells. Transgenic mice expressing IκB-γ will be generated, and to determine if B cell differentiation is blocked in these mice, the number of peripheral B cells expressing surface IgM will be measured using FACS (fluorescence-activated cell-sorting) analysis. Finally, to determine if the expression of IκB-γ causes neoplasia, pathological analysis of peripheral blood, spleen, and bone marrow will be performed.

Dr. Ghosh is also Assistant Professor of Immunobiology and of Molecular Biophysics and Biochemistry at Yale University School of Medicine.

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SELF-TOLERANCE MECHANISMS IN B LYMPHOCYTES

Christopher C. Goodnow, B.V.Sc., Ph.D., *Assistant Investigator*

Production of autoantibodies is characteristic of most autoimmune diseases and is normally avoided by censoring of self-reactive cells within both the B cell and helper T cell repertoires. Research in Dr. Goodnow's laboratory focuses on the B cell repertoire and has three major aims: 1) definition of cellular and molecular events responsible for censoring self-reactive B cells *in vivo*, 2) development of new mouse models for analyzing B cell tolerance or autoimmunity to different types of self antigens, and 3) determination of the basis for failure of B cell tolerance in strains of autoimmune mice.

Elimination of Self-reactive B Cells

To visualize the development and fate of self-reactive B lymphocytes *in vivo*, Dr. Goodnow's labo-

ratory makes use of transgenic mice carrying rearranged immunoglobulin (Ig) genes. Because the rearranged Ig transgenes prevent endogenous Ig gene rearrangement, most of the developing B cells in these mice express a single specificity of Ig molecules as cell surface antigen receptors and as secreted antibodies. Thus mice carrying Ig genes encoding an anti-lysozyme antibody contain a large number of hen egg lysozyme (HEL)-specific B cells that can be easily followed and analyzed.

During the past year, members of Dr. Goodnow's laboratory have been studying one mechanism of B cell censoring that involves physical elimination of self-reactive B cells. Elimination of lysozyme-specific B cells could be clearly shown to occur in offspring from matings between anti-HEL Ig-

transgenic mice and mice carrying a transgene that encodes a membrane-bound form of lysozyme (mHEL).

In the double-transgenic offspring resulting from these matings, anti-HEL B cells encountered "self" mHEL on the surface of neighboring stromal and hematopoietic cells within the bone marrow, and as a result failed to appear as mature recirculating B cells in the peripheral lymphoid organs. The aborted development of self-reactive B cells in these mice contrasts with the normal development of self-reactive cells observed previously in double-transgenic mice expressing soluble lysozyme (sHEL; see below), implying that extensive receptor cross-linking by multivalent ligands such as cell surface molecules may be necessary for triggering B cell elimination.

More recent studies by Suzanne Hartley and Dr. Michael Cooke have focused on defining cellular events leading to B cell elimination. In theory, the simplest explanation for the failure to detect mature B cells in these mice is that receptor crosslinking had directly induced a programmed cell death pathway. Analysis of bone marrow from the double-transgenic mice, however, revealed that the pool of immature self-reactive B cells that was first being exposed to mHEL was not in the process of cell death. FACS (fluorescence-activated cell sorting) and culture of these cells revealed that they had also not become committed to cell death, but could mature and be triggered into antibody production if removed from further exposure to mHEL. Instead, the immediate consequence of continuous receptor crosslinking *in vivo* and *in vitro* was to arrest further development of the immature self-reactive B cells.

The failure of the self-reactive B cells to mature and express a number of important receptor molecules, such as the lymph node homing receptor, complement receptors, and CD23, may in turn cause cell death by default. To test further the notion that cell death was secondary to arrested development, transgenic animals carrying the Ig transgenes and a *bcl-2* transgene were produced, using *bcl-2*–transgenic mice provided by Drs. Alan Harris and Suzanne Cory (Walter and Eliza Hall Institute, Melbourne). The *bcl-2* transgene markedly delayed death and elimination of self-reactive B cells in mHEL-expressing mice but failed to perturb the more immediate process of arrested development.

The significance of these findings is severalfold. First, the identification of proximal regulatory events in the elimination pathway should facilitate efforts to identify the underlying molecular mechanisms. Second, the potential reversibility of the early arrested phase of B cell elimination may contribute to escape of self-reactive B cells from elimination in autoimmune diseases. Both issues are now being explored.

Inactivation of Self-reactive B Cells

In contrast to the fate of self-reactive B cells in double-transgenic mice expressing membrane-bound HEL, Dr. Goodnow and his colleagues previously found that anti-HEL B cells of double-transgenic mice expressing a secreted form of HEL were not eliminated. In this case, the self-reactive B cells were nevertheless functionally silenced, in that they were incapable of mounting an effective antibody response *in vivo*. This phenomenon of functional silencing, or so-called clonal anergy, may represent a second, general mechanism for censoring self-reactive cells in both B and T cell repertoires.

Defining the basis for functional silencing in self-reactive B cells has formed a focus of research conducted by Drs. Sarah Bell and Michael Cooke. Studies employing alloreactive helper T cells to promote B cell proliferation *in vivo* and *in vitro* have firmly established that the tolerant B cells are stimulated into clonal expansion much less efficiently than nontolerant controls. B cell proliferation under these conditions has been found to depend on lysozyme binding to the B cell antigen receptor in addition to signals from helper T cells.

In collaboration with Drs. Andrew Heath and Maureen Howard (DNAX Inc., Palo Alto), signaling through two of the most important molecules responsible for receiving T cell help, namely the interleukin-4 receptor and CD40, has been shown to occur normally in the tolerant cells. By contrast, signaling through the B cell antigen receptor is markedly altered in the tolerant B cells, and this appears likely to account for their inefficient proliferation. Determining the molecular basis for the changes in antigen receptor signaling in tolerant B cells has therefore become a primary focus of this work.

Development of New Mouse Models

A number of important autoimmune diseases are caused by production of autoantibodies to tissue-specific receptors, such as the thyroid-stimulating hormone receptor or the acetylcholine receptor. To develop mouse models for studying B cell tolerance and autoimmunity to these and other types of tissue-specific self antigens, Dr. Goodnow and his colleagues have begun producing new lines of HEL-transgenic mice. Srinivas Akkaraju has prepared a number of different gene constructs with different tissue-specific promoters and coding regions and

has begun to analyze the first lines of transgenic mice where lysozyme expression should be targeted to the thyroid gland. (This project has been supported by a grant from the National Institutes of Health.)

Dr. Goodnow is also Assistant Professor of Microbiology and Immunology at Stanford University School of Medicine.

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MODELS OF COMPLEMENT RECEPTOR AND REGULATORY PROTEIN FUNCTION

V. Michael Holers, M.D., *Assistant Investigator*

Complement proteins and their activation fragments play a central role in host defense against foreign antigens. Fragments of activated complement bound to targets mediate their attachment by specific receptors to cells of the immune system. Secondary effects on cells bound by these targets via complement receptors include changes in intracellular calcium, phosphorylation of specific substrates, cell adhesion, and the transcriptional activation state of specific genes.

Fragments of activated complement may also deposit randomly on self membranes rather than on desired targets. To protect against this, intrinsic cell membrane proteins have evolved with a primary role of inactivating these self membrane-bound fragments.

Complement receptors and membrane regulatory proteins that mediate both attachment of complement-bound targets and the control of inappropriate deposition are part of a large family called the regulators of complement activation (RCA). The genes encoding this protein family are found at a single locus on human chromosome 1q32. The structure of the members of this family consists of a related 60– to 70–amino acid repeat, designated a short consensus repeat, containing four conserved cysteines and one conserved tryptophan. Analysis of the genomic structures of this family has suggested derivation from a common element.

Currently both the structure-function relationships of these proteins and the phenotypic changes resulting from the attachment of complement-bound fragments to their receptors on cells of the immune system are being investigated. Dr. Holers has concentrated most of his efforts during the past three years on human complement receptor 2 (CR2), which is the receptor for a complement component C3 fragment designated C3d, in addition to being the receptor for the Epstein-Barr virus (EBV). More recently Dr. Holers has begun to establish mouse models of RCA protein family activities in order to study their roles in specific immune responses.

Human CR2

Human CR2 is an ~145-kDa B cell receptor consisting structurally of 15 or 16 short consensus repeats followed by transmembrane and short intracytoplasmic domains. Recently Dr. Holers has focused on two areas in regard to CR2. First, the structural determinants on the molecule that mediate binding to C3 and EBV are being probed by a combination of mutagenesis and peptide-based methods. Using these techniques, he has identified a number of peptide sequences in CR2 that are likely to be contact sites for C3 and EBV binding. (This project is supported by funds from the National Institutes of Health.)

Dr. Holers is also studying the ability of CR2 to regulate cell-cell adhesion when bound by its ligands. He is determining the structural characteristics of CR2 that influence its ability to mediate this type of adhesion. In addition, he is analyzing the signaling pathways involved. His studies suggest

that ligand binding to CR2 may play a substantial role in the control of specific cellular adhesion pathways. These events are likely to figure importantly in the various mechanisms by which complement regulates the immune response.

Mouse Homologues of Human Complement Receptor and Regulatory Proteins

Recently Dr. Holers and his colleagues have identified three mouse homologues of human RCA proteins. In these studies the group has determined that there are interesting structural differences in the receptors and in the mechanism of cell-specific regulation of expression between the human and mouse RCA family members. Despite the differences, however, there are very similar, if not identical, functional activities.

Each of the three homologues demonstrates this point. The first two are products of an alternatively spliced mRNA that, in the mouse, encodes the homologues of two unique human RCA proteins, complement receptor 1 (CR1) and CR2. The third is a novel protein, Crry/p65. This protein is a mouse functional homologue of two human RCA proteins: decay-accelerating factor and membrane cofactor protein. Dr. Holers has demonstrated that Crry/p65 has the same biologic activity—control of complement deposition on self membranes—as these two human proteins. By the construction of appropriate monoclonal and polyclonal reagents, he is characterizing in each tissue the cell-specific expression of Crry/p65.

His studies on this protein have suggested that within the RCA family an evolutionary switch has occurred that has resulted in a mouse protein with little structural similarity to decay-accelerating factor or membrane cofactor protein but with very similar if not identical biologic activities. Ongoing studies are characterizing the roles of these proteins in the immune response to foreign antigens and in autoimmune disease.

Regulation of Human CR2 Expression

Human CR2 is expressed primarily on B lymphocytes in a stage-specific manner. It is not expressed on early pre–B cells, is expressed on mature circulating and germinal center B cells, but is not found on immunoglobulin-secreting later-stage B cells or plasma cells. Thus CR2 serves as a model of regulation of stage-specific proteins in this cell type.

Dr. Holers and his colleagues are currently analyzing the transcriptional regulatory domains within this gene. He has determined that control of surface expression is primarily related to transcriptional activation. The laboratory has identified a series of functionally important areas within the human CR2 promoter and a number of transactivating protein families involved in this regulation. He is currently determining which of these are important in cell- and stage-specific expression of CR2. The overall goal is to use this information to further an understanding of the molecular genetic mechanisms by which B lymphocytes mature and are activated.

Dr. Holers is also Associate Professor of Medicine and Assistant Professor of Pathology at Washington University School of Medicine and Assistant Physician at Barnes Hospital, St. Louis.

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MOLECULAR STRATEGIES TO CONTROL TUBERCULOSIS AND DEVELOP NOVEL VACCINES AGAINST THIRD WORLD DISEASES

WILLIAM R. JACOBS, JR., PH.D., *Assistant Investigator*

Tuberculosis, caused by *Mycobacterium tuberculosis*, has reemerged in the United States as a tremendous public health problem, with increasing numbers of new cases for the last six years. In addition, *M. tuberculosis* strains have emerged that are resistant to all known chemotherapeutic agents. Worldwide, over 8 million new cases of tuberculosis are reported each year, since the disease has remained a problem throughout the developing world. The 3 million deaths it causes annually make it the leading infectious killer in the world today.

Dr. Jacobs' laboratory uses molecular genetic approaches to investigate the biology of *M. tuberculosis*.

Epidemiological Analysis of Tuberculosis Infections

Restriction fragment length polymorphism (RFLP) analysis can play a key role in determining the mode of transmission of tuberculosis, as individual isolates can be tracked from one infected individual to the next. The recent increases in the incidence of tuberculosis in the United States seem to be associated with the AIDS epidemic. However, it is unclear whether the tuberculosis of AIDS patients results from reactivated disease, reflecting an exposure to the tubercle bacillus prior to infection with AIDS, or from a first-time infection. In collaborations with others, including Drs. Peter Small and Gary Schoolnik (HHMI, Stanford University), analyses were undertaken to distinguish between these two possibilities.

Different isolates of *M. tuberculosis* have different RFLP patterns when probed with a particular DNA element found in *M. tuberculosis* strains. RFLP analysis of the strains isolated from a recent tuberculosis outbreak among AIDS patients in a group home revealed that 11 individuals had all been infected with the identical strain of *M. tuberculosis*. This demonstrates that AIDS patients are highly susceptible to infection with *M. tuberculosis* and confirms that tuberculosis is highly contagious. The clear results of these studies should be translatable into better public health care policies. Similar analyses are under way to track *M. tuberculosis* isolates that are multiply drug resistant.

Genetic Analysis of Mycobacterial Virulence Determinants

By understanding how mycobacteria infect and cause disease in mammalian hosts, it should be possible to develop effective measures to treat and prevent mycobacterial disease. One approach to understanding the virulence determinants of *M. tuberculosis* is to identify and characterize the genes responsible for the virulence phenotype. Gene function, such as virulence, can be defined by generating well-defined mutants and comparing the mutant strain with the wild-type bacterium. Dr. Jacobs' laboratory has focused on developing methods to generate well-defined mutations in mycobacteria.

Methods of mutagenesis using chemical or physical agents to damage DNA, coupled with the screening of colonies arising from single clones of cells, are particularly unsuitable for the mycobacteria. The organisms grow in clumps, not as single-cell suspensions, as a result of their complex cell wall structure. Insertional mutagenesis would be a more effective way of mutagenizing mycobacteria, as this method most often employs a transposon that contains a selectable marker gene. Colonies of mutated cells are selected for by plating cells in the presence of a selecting agent. The agent prevents the growth of the nonmutagenized cells clumped to cells that contain a transposon, and thus the colonies that arise are a pure population of a mutagenized clone.

When Dr. Jacobs' laboratory initiated this work, no useful transposons that functioned in mycobacteria had been identified. In the absence of a transposon, a shuttle mutagenesis approach was successfully employed. This involved the cloning of DNA fragments from *Mycobacterium smegmatis*, BCG (bacille Calmette-Guérin), and *M. tuberculosis* into vectors that could not replicate in mycobacteria. Transposons were introduced into the cloned mycobacterial DNA fragments in *Escherichia coli*. Then the transposon-mutated DNA fragment was reintroduced into the mycobacterial chromosome via homologous recombination of the DNA fragments on the adjacent sides of the transposon.

This system was useful for generating mutations in both *M. smegmatis* and *M. tuberculosis*. Surprisingly, however, analyses revealed that *M. tuberculosis* displayed a high degree of illegitimate recombination as compared with *M. smegmatis* and that the illegitimate recombination process was not terribly efficient.

Serendipitously, a novel insertion element, IS*1096*, was discovered in Dr. Jacobs' laboratory. IS*1096* has been demonstrated to possess many properties that make it attractive as a potential insertional mutagen of *M. tuberculosis*. It is small, it

hops in a random fashion, and it is not found in *M. tuberculosis* strains. Current work is under way to characterize IS*1096* genetically and use it as the source of a novel transposon for random mutagenesis of mycobacteria. (The project described immediately above was also supported by a grant from the National Institutes of Health.)

Recombinant BCG Vaccines and Novel Vaccine Strategies

BCG is an attenuated mutant of the bovine tuberculosis bacillus that has been used as a vaccine against tuberculosis in humans for over 50 years. This bacterium possesses several unique properties that make it an ideal candidate for use as a live vaccine vector for generating multivalent vaccines. It is proven safe, having been used in 2.5 billion individuals with a significantly lower mortality rate than smallpox vaccine. Currently it is the only live vaccine other than oral polio that is recommended by the World Health Organization to be given at birth. Also, the mycobacterial cell wall possesses potent adjuvant properties that can engender excellent humoral responses. In addition, since BCG normally resides within macrophages that are key antigen-presenting cells, it follows that it can elicit cellular immune responses to associated antigens.

In the past few years, Dr. Jacobs' laboratory, in collaboration with Drs. Barry Bloom (HHMI, Albert Einstein College of Medicine), Graham F. Hatfull (University of Pittsburgh), and C. Kendall Stover (MedImmune), has developed a series of expression vectors and transformation systems that make it possible to clone and express in BCG antigen-encoding foreign genes from virtually any pathogen. Mice immunized with these recombinant BCG cells expressing foreign antigens have been shown to elicit both humoral and cellular immune responses to the expressed foreign proteins.

To investigate the immune responses to foreign proteins expressed by BCG, Dr. Jacobs' laboratory is currently cloning and expressing genes from organisms that cause leishmaniasis, schistosomiasis, filariasis, and toxoplasmosis. Such analyses should provide the basic knowledge needed to engineer a recombinant BCG that could protect humans from these dread diseases.

Dr. Jacobs is also Associate Professor in the Departments of Microbiology and Immunology and of Molecular Genetics at Albert Einstein College of Medicine of Yeshiva University, Bronx.

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ACTIVATION OF THE CD4 T CELL

Charles A. Janeway, Jr., M.D., *Investigator*

Studies in Dr. Janeway's laboratory focus on the activation of CD4 T cells in normal adaptive immune responses as well as in selected animal models of autoimmune disease. Studies cover several aspects of the basic process of CD4 T cell activation that have application to the disease models.

The Ligand for the CD4 T Cell Receptor

CD4 T cells recognize a molecular complex consisting of a peptide fragment of antigen bound in the groove of a class II molecule of the major histocompatibility complex (MHC). This complex ligand has been inferred from many studies, but only recently has it been characterized directly. Dr. Janeway's group has isolated and sequenced several naturally processed peptide fragments bound to the groove of several different MHC class II molecules. These dominant peptides are distinct in structure from those isolated from MHC class I molecules by other laboratories, being longer by several amino acids and differing in length at both ends. These length differences, however, do not markedly affect the specificity of T cell recognition, which is focused on the central region of the peptide.

The peptides show specific residues at two or more positions within the sequence, and these are likely involved in binding to MHC molecules. The data are compatible with the groove on the MHC class II molecule being similar to that of MHC class I, but differing at the ends, where MHC class II molecules are likely to be open, allowing the bound peptide to extend out at one or both ends. The T cell receptor (TCR) seems likely to focus only on the central region of 8–10 amino acids that includes MHC-binding residues and those that interact with the TCR central region. The technique used by Dr. Janeway's group should provide a novel means of identifying major peptide epitopes in infectious diseases and autoimmunity, and this is being tested in *Salmonella typhimurium* infection and in diabetes-prone mice. Moreover, the definition of peptide motifs by direct sequencing of MHC class II–associated peptides provides an alternative approach to searching for peptide epitopes within proteins. (A grant from the National Institute of Allergy and Infectious Diseases, National Institutes of Health, provided partial support for this project.)

Rules for Antigen Presentation by MHC Class II Molecules

Based on the isolation of peptides from MHC molecules and other studies, several rules that govern presentation of peptides by MHC class II molecules have been derived. These are as follows. First, the peptide donor must be abundant in the processing compartment; membrane-associated proteins that are internalized appear to be particularly attractive peptide donors. Second, the peptides have a variable length, with a mean of 16 amino acid residues and a range of 13 to 22 residues in the hands of the Janeway laboratory. Third, the peptide must have certain amino acids at particular positions to allow it to bind stably to the MHC class II molecule that will transport it to the cell surface. Finally, the peptide must be producible from the protein in the processing compartment.

Dr. Janeway and his colleagues have discovered a self protein whose carboxyl-terminal peptide is potently immunogenic in mice; that is, the mice are not tolerant to the self peptide. T cells specific for the peptide do not recognize the parent protein, apparently because of a failure to generate the peptide. The same peptide from the foreign homologue, differing by two amino acids from self, is readily processed from the native protein. Thus a protein must be able to yield the peptide in order for that peptide to be recognized. Working out the rules for processing using this system will be very interesting.

Distribution of Dominant Peptide Epitopes and Its Implications for T Cell Development

One of the central mysteries of immunology is the selection of the repertoire of TCRs. From a random, clonally distributed repertoire expressed by early developing T cells in the thymus, only those that can recognize peptides presented by self MHC molecules mature. Moreover, potentially harmful T cells recognizing self peptides bound by self MHC molecules are deleted from this surviving pool. Thus mature T cells are self MHC restricted and self tolerant. The puzzle is how a receptor interacting with self MHC molecules binding self peptides can signal for positive selection at one stage of development and then for negative selection at a later phase. Such a system would seem to leave no cells available for export. Thus there must be some difference in the response of the cell to the same signal, or in the ligand itself.

Having identified self peptides bound to self MHC molecules, Dr. Janeway's laboratory has used a unique monoclonal antibody that recognized one such complex to examine its abundance and distri-

bution. It was found that this complex of self peptide and self MHC makes up one-eighth of all complexes on cells involved in CD4 T cell tolerance or activation. However, this complex is present at very low levels on thymic epithelial cells, the cells that provide the MHC ligands involved in positive selection. In mice having either thymic epithelial cells or bone marrow–derived cells expressing this self peptide:self MHC complex, one finds tolerance only when the self peptide is expressed on bone marrow–derived cells.

Moreover, there appears to be excess positive selection by the low level of this peptide found on thymic cortical epithelium in two different systems. This suggests that positive selection involves interactions of the TCR with ligands at low density, while negative selection involves contact with antigen-presenting cells expressing higher levels of a limited repertoire of self peptides. This hypothesis is in keeping with the low density of TCRs on thymocytes prior to positive selection and with the low density of MHC class I molecules on thymic cortical epithelium.

Orientation of the TCR Relative to Its MHC Ligand

There has been much speculation about the nature of the crucial interaction in clonal selection of CD4 T cells—the binding of the peptide:MHC class II complex by the TCR. Dr. Janeway's laboratory has generated chimeric TCRs by genetic engineering. These studies have shown that the centrally placed hypervariable regions are required for all responses of a cloned T cell line. However, variable amino acids that map to the first and second complementarity-determining regions of the TCR α chain are also involved in the fine specificity of MHC recognition by this cloned T cell line.

Exchanging these regions between two TCRs generates novel responses not observed with either parental line alone. The critical interaction is between the helical region of the α chain of the MHC class II molecule and the Vα-encoded first and second hypervariable regions of the TCR. Resolution of this interaction by site-directed mutagenesis of the TCR and the MHC class II molecule is nearly complete and should provide a detailed picture of the orientation of these two structures during antigen recognition. To confirm this, the structure of the relevant molecules must be determined. In collaboration with Dr. Alfred Bothwell, soluble receptors have been prepared from this cloned T cell line for structural analysis. A major goal of the coming year will be the analysis of these soluble molecules. (This project was supported in part by a grant from the National Institute of Allergy and Infectious Diseases.)

Co-stimulation Is Also Required to Activate CD4 T Cells

Activated CD4 T cells can respond directly to ligand alone by producing cytokines that mediate effector functions. However, the initiation of an immune response requires clonal expansion of naive CD4 T cells through the production of the T cell growth factor interleukin-2 (IL-2). To produce IL-2, a naive T cell needs to receive co-stimulatory signals in addition to receptor ligation. Dr. Janeway's laboratory has shown that one cell must present both ligand and co-stimulator in order to induce clonal expansion of normal CD4 T cells. At least two co-stimulatory molecules that act in concert have been defined, the B cell activation antigen B7/BB1 and the heat-stable antigen. Many distinct microbial constituents will induce expression of co-stimulatory activity on antigen-presenting cells. Dr. Janeway has proposed that recognition of invariant microbial components by primitive immune recognition systems triggers the expression of co-stimulatory activity, allowing the adaptive immune response to distinguish infectious non-self from non-infectious self. The definition of these recognition systems is a major future goal of the laboratory. (This project was supported in part by a grant from the National Institute of Allergy and Infectious Diseases.)

Homing of Activated Effector Cells to Tissues

When naive CD4 T lymphocytes encounter antigen in lymph nodes or spleen, they must first proliferate and then differentiate into active effector cells. These cells must leave the lymphoid organs and home to sites of infection in order to mediate their functions. They leave the lymphoid organs by efferent lymphatics and enter the blood, where they circulate to all parts of the body. However, they are only useful if they can enter the site of infection. Dr. Janeway's laboratory has asked what molecules are involved in the homing of effector cells to potential infection sites.

The β_1-integrin VLA-4 appears to be crucial for this step, binding to VCAM-1 on endothelial cells. In the system studied, activated T cells specific for a brain antigen would enter normal brain only if they express VLA-4, and entry was VLA-4 and VCAM-1 dependent. Dr. Janeway believes that VLA-4 and other adhesion molecules that are up-regulated on effector T cells allow the cells to leave the vessels and enter tissues. If antigen or infection is present, then the cells respond and recruit other cells to mediate

elimination of the pathogen. If antigen is not present, then the effector cells either enter afferent lymphatics and thus return to the blood, or they die in the tissues. As Dr. Janeway's studies actually used cells reactive to a self antigen, similar considerations also apply to the homing of autoreactive T cells to tissues and would presumably apply to graft rejection as well.

Insulin-Dependent Diabetes Mellitus, a Model Autoimmune Disease

CD4 T cells play a crucial role in autoimmune diabetes, which Dr. Janeway's laboratory is studying through use of the spontaneous diabetes seen in the nonobese diabetic (NOD) mouse. Cloned islet-specific CD4 T cells are required to transfer disease, but only if cloned islet-specific CD8 T cells are also transferred. More recently Dr. Janeway's laboratory has shown that islets of diabetic mice contain not only T cells that can destroy pancreatic β cells, causing diabetes, but also CD4 T cells that can transfer protection against diabetes. These protective T cells are of great interest, as their specific activation might be a means of disease prevention. Thus such cells are currently under intensive study.

A second area of study is the protection afforded NOD mice by the MHC class II I-E transgene. Dr. Janeway, in collaboration with the laboratory of Dr. Richard Flavell (HHMI, Yale University), has shown that this protection requires I-E expression on peripheral antigen-presenting cells. These cells play a role in inducing self tolerance, and it is interesting in this regard that the cloned T cell lines that transfer diabetes express TCRs encoded in Vβ1 gene segments. T cells bearing such receptors are prominent in prediabetic islets and have a restricted set of sequences, suggesting a role for specific receptors in this disease. Whether T cells of this type are deleted by I-E expressed on peripheral antigen-presenting cells is now being tested, as this could provide a simple explanation of I-E's protective effect. Finally, the superantigen SEA (staphylococcal enterotoxin A) accelerates diabetes in NOD mice and also stimulates T cells expressing Vβ1-encoded receptors. Thus a careful analysis of Vβ1 expression is another focus of this program. (This work is supported in part by a grant from the National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health.)

The NOD mouse shares a unique feature of its MHC class II molecules with most human diabetics: the absence of an aspartic acid residue at position 57 on the β chain. To examine the nature of peptides bound to NOD MHC class II molecules, Dr. Janeway and his colleagues have purified this complex,

eluted peptides, and sequenced them. These peptides show a distinctive peptide motif, one feature of which is an acídic residue near the carboxyl terminus. Peptides eluted from three other MHC class II molecules that have aspartic acid at position 57 of the β chain do not have an acidic residue at this site. Thus a unique islet autoantigen may bind much better to non–aspartic 57 MHC class II molecules because the peptide has an acidic residue that is prevented from binding by the aspartic acid at residue 57. This hypothesis is being tested by site-directed mutagenesis, synthetic peptide binding, and further peptide-sequencing studies.

In summary, studies in Dr. Janeway's laboratory focus on the activation of CD4 T cells, from the generation of ligands and co-stimulatory molecules on antigen-presenting cells through the precise nature of TCR binding to these complexes, to more difficult questions of autoimmune disease and lymphocyte homing. They seek to explain the phenomena of adaptive immunity in T cells and its failure in autoimmune disease.

Dr. Janeway is also Professor of Immunobiology at the Yale University School of Medicine and of Biology at Yale University.

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THE T CELL RECEPTOR AND ITS LIGANDS

JOHN W. KAPPLER, PH.D., *Investigator*

Normally an effective response by the immune system's T cells to the antigens of microbial invaders is essential to preventing and curing infection. However, Dr. Kappler and his colleagues have discovered that for a class of microbial protein antigens that they call superantigens, a T cell response appears to be detrimental to the host. The key feature of these special antigens is that T cells respond to them more frequently by many orders of magnitude than they do to conventional antigens.

T cells recognize foreign antigens by means of a surface receptor that varies from one T cell to the next. The five variable components of the receptor are called $V\alpha$, $J\alpha$, $V\beta$, $D\beta$, and $J\beta$. Usually fragments of foreign antigens create a ligand for this receptor when they bind to a groove on one of the cell-bound products of the major histocompatibility complex (MHC). The frequency of T cells responsive to this antigen-MHC complex is low ($\sim 0.001\%$), since the interaction requires exactly the right combination of the five receptor components to generate a match.

T cells also recognize superantigens complexed to MHC molecules. But the superantigens appear to bind to a unique site on the MHC in such a way that only the $V\beta$ portion of the T cell receptor need be of the correct type to generate a response. This results in a high frequency of responding T cells (1–10%). The activation of so many T cells can be disastrous for the host because of the sudden release of powerful biological mediators. Symptoms include vomiting, diarrhea, toxic shock, and even death. Dr. Kappler and his colleagues have been examining the superantigens' structural features that underlie their unusual properties. They have found that the mode of action of superantigens from different types of

microorganisms is similar, but the proteins are much different in structure.

A well-defined group of superantigens are the exotoxins produced by streptococcal and staphylococcal bacteria that cause food poisoning and toxic shock syndrome. These toxins are secreted globular proteins. Dr. Kappler's group has studied one of them in detail, staphylococcal enterotoxin B (SEB). They identified amino acids, especially in the amino-terminal region, that controlled the toxin's ability to bind to MHC molecules. Other amino acids in the same portion of the molecule were found to control the interaction with the Vβ portion of the T cell receptor.

Dr. Kappler's group has also studied a set of superantigens that they found to be encoded by a gene (*vsag*) in the 3'LTR (long terminal repeat) of mouse mammary tumor viruses (MTVs). The viruses are passed in the milk from mother to pup. The retrovirus appears to use these superantigens to create a large set of activated T cells (and indirectly B cells) that the virus can infect while awaiting the maturation of its ultimate target tissue, the mammary gland. The viral superantigens have no sequence homology with the bacterial ones, and their structures are much different. Through use of biochemical techniques and monoclonal antibodies specific for the viral superantigens, the proteins were shown to be type II integral membrane proteins with the unusual property of an extracellular carboxyl-terminal region. An examination of these *vsag* genes from a large set of infectious and integrated proviral MTVs revealed an extraordinary heterogeneity in sequence and length of the protein's 20–30 amino acids in the carboxyl-terminal region, correlating with Vβ specificity.

An extensive analysis of the site of interaction between superantigens and Vβ elements of the T cell receptor has been performed. Based on the similarities between T cell receptors and antibodies, these results indicate that both bacterial and MTV superantigens interact with Vβs on a solvent-exposed site facing away from the site that interacts with conventional peptide antigens bound to MHC. Thus the bacterial and viral superantigens seem to be an unusual example of convergent evolution in which two proteins have evolved to have the same function and occupy proximate sites, but apparently with very little structural homology. (This work has received support from the National Institutes of Health.)

Dr. Kappler is also a member of the Division of Basic Immunology of the Department of Medicine at the National Jewish Center for Immunology and Respiratory Medicine, Denver, and Professor of Microbiology and Immunology and of Medicine at the University of Colorado Health Sciences Center, Denver.

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MOLECULAR GENETICS OF LYMPHOCYTE DEVELOPMENT

Stanley J. Korsmeyer, M.D., *Associate Investigator*

The *Bcl-2* Proto-oncogene

The t(14;18) chromosomal breakpoint found in follicular B cell lymphoma juxtaposes the new proto-oncogene *Bcl-2* with the immunoglobulin (Ig) heavy-chain gene, resulting in marked overproduction of Bcl-2. *Bcl-2* is novel among proto-oncogenes in that it localizes to mitochondria and demonstrates the unique role of blocking programmed cell death without promoting cell division.

Transgenic mice bearing a *Bcl-2*–Ig minigene, which recapitulates the t(14;18), initially display a polyclonal expansion of resting B cells with a prolonged life span. Over time these mice develop monoclonal, high-grade lymphomas. Extended cell survival can be a primary oncogenic event that appears to favor the acquisition of secondary genetic abnormalities.

Bcl-2 protein is widespread during embryonic development but topographically restricted in adult tissues. Within adult tissues that demonstrate apoptotic cell turnover, Bcl-2 is often geographically restricted to long-lived or proliferating cell zones. Many developing organs express Bcl-2 at high levels and in a more widespread pattern. For example, the relatively undifferentiated neuroepithelium in the mouse retina at embryonic day 14.5 uniformly expresses Bcl-2. Following differentiation and migration, the inner nuclear layer of interneuron is much more intense than the outer layer of photoreceptors. At day 11.5, Bcl-2 is present throughout the embryonic limb bud, but by day 14.5, when digits have separated, expression is lost in the interdigital regions of death. The distribution pattern of Bcl-2 suggests that it is selectively downregulated to enable cell death to occur.

Maintenance of B cell memory represents a normal physiologic role for Bcl-2. Bcl-2–Ig mice that overproduce Bcl-2 provide an *in vivo* model to assess the role of Bcl-2 upon immune responsiveness. Secondary immune responses by these transgenics were markedly protracted. This resulted from long-term persistence of antibody-secreting plasma cells as well as an extended lifetime for resting memory B cells. Bcl-2 spared the need for antigen in maintaining immune responsiveness.

Bcl-2 inhibits multiple forms of apoptosis but not negative selection within thymocytes. In the thymus, Bcl-2 is present in the mature T cells of the medulla but only in rare cells of the cortex. To assess the role of Bcl-2 in the programmed death of thymocytes, *lck^{Pr}*–*Bcl-2* transgenic mice were generated that redirected Bcl-2 expression to cortical thymocytes. Bcl-2 protected immature CD4$^+$CD8$^+$ thymocytes from glucocorticoid, radiation, and anti-CD3–induced apoptosis. Moreover, Bcl-2 alters T cell maturation, increasing CD3^{med} thymocytes felt to represent an intermediate stage after positive selection. Despite this, clonal deletion of T cells that recognize endogenous superantigen still occurred.

Moreover, matings with T cell receptor transgenic mice indicate that the negative selection of thymocytes reactive with class I– or class II–restricted antigen also occurred. Such double-transgenic mice, when placed on a neutral MHC background, suggest that Bcl-2 may participate in positive selection. It appears that multiple death pathways operate within the thymus and can be distinguished by their dependence on Bcl-2.

A novel negative regulatory element inhibits expression from an upstream promoter. Nuclear run-on assays demonstrate that *Bcl-2* transcription decreases in parallel with RNA levels during B cell differentiation. Promoter-reporter constructs identified a negative regulatory element (NRE) in the *Bcl-2* 5′-untranslated region. The NRE dramatically decreases expression from the *Bcl-2* P1 promoter or heterologous promoters in a position-dependent fashion.

Multiple functionally redundant sequence elements mediate NRE activity. Chromatin structure of the endogenous NRE differs from pre-B to mature B lines. These results indicate that negative control of transcription is an important determinant of the differential expression of *Bcl-2*.

Transgenic Mice That Redirect Transcription Factors to the Thymus Develop T Cell Acute Lymphoblastic Leukemia/Lymphoma

Interchromosomal translocations within T cell neoplasms often involve their antigen receptor genes and another chromosome that provides a new transcription factor gene. *Ttg-1*, from chromosome segment 11p15, encodes a nuclear protein—predominantly expressed in neuronal cells—and belongs to a novel family of transcription factors with LIM (*lin-11*, *Isl-1*, *mec-3*) domains.

Transgenic mice that overexpress this candidate oncogene in early thymocytes develop immature, aggressive T cell leukemia/lymphoma. A minority

population of thymocytes representing an immature $CD4^-8^+$ intermediate stage is preferentially affected. This model indicates that aberrant expression of putative transcription factors plays a primary role in the genesis of T cell acute lymphocytic leukemia (ALL).

HOX11 is a novel homeobox discovered in T cell ALL at the site of the t(10;14). In normal tissues it is expressed in embryonic brain but is not avidly expressed in thymus or in resting or activated T cells. Some lines of transgenic mice that overexpress HOX11 in thymus show perturbed T cell development. Total thymocytes are reduced to <10%, with marked diminution in $CD4^+8^+$ double-positive and single-positive cells. The S phase percent is normal in immature $CD3^-$ thymocytes, but is markedly increased to 30% with maturation.

Transgenic thymocytes demonstrate accelerated apoptosis. To date, 33% (9/27) of transgenic mice with an abnormal phenotype developed T cell lymphoma, while none (0/64) with a normal phenotype developed lymphoma. The mice provide a model to dissect the role of altered cell cycle progression and programmed cell death in oncogenesis.

The t(4;11) Breakpoint Found in Aggressive ALL Generates a Novel Fusion Transcript

Chromosome segment 11q23, a common breakpoint in mixed-lineage leukemias, includes the t(4;11) of preB/monocytic ALL. As these breakpoints were distant from known genes, yeast artificial chromosome (YAC) technology was employed. A 280-kb YAC of 11q23 origin was characterized and shown to span the breakpoints. Topoisomerase II–binding sites and χ-like sequences were noted near the breakpoint, suggesting a role for these elements in the translocation.

Leukemias bearing a t(4;11) demonstrate an abnormal-sized transcript. Fusion cDNAs from the der(11) predict a chimeric protein composed of centromeric 5′ sequences from 11q23 and telomeric 3′ sequences from 4q21. The juxtaposition of two newly discovered genes creates a fusion protein novel to these poor-prognosis leukemias.

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FUNCTION OF T CELL SURFACE GLYCOPROTEINS IN DEVELOPMENT AND IN HIV PATHOGENESIS

DAN R. LITTMAN, M.D., PH.D., *Associate Investigator*

Research in Dr. Littman's laboratory has focused on understanding the molecular events involved in T lymphocyte differentiation and activation and in the processes through which human immunodeficiency virus (HIV) enters target cells and causes systemic depletion of helper T cells. Both areas of investigation involve studies on the functions of T cell surface molecules and on their interactions with intracellular signal transducing components.

Signal Transduction in Thymocyte Differentiation and T Cell Activation

Mature T cells that emerge from the thymus are tolerant to self antigens yet react to foreign antigens complexed to host major histocompatibility complex (MHC) molecules. Selection of T cells bearing the appropriate T cell receptors (TCRs) involves deletion of cells specific for self antigen and expansion (positive selection) of clones that react with host MHC molecules.

In the course of development, precursor thymocytes that express both the CD4 and CD8 cell surface glycoproteins (double-positive cells) give rise to mature $CD4^+$ T helper cells specific for class II MHC molecules and $CD8^+$ cytotoxic cells that recognize class I MHC molecules. The CD4 and CD8 molecules have been shown to bind to membrane-proximal domains of class II and class I MHC molecules, respectively. It has been proposed that signals transmitted through the CD4 and CD8 molecules, possibly via the associated cytoplasmic tyrosine kinase $p56^{lck}$, are important in clonal deletion, positive selection, and specification of the developmental pathways of double-positive cells.

To study these problems, mice lacking expression of CD4 were generated by disruption of the CD4 gene in an embryonal stem cell. Animals homozygous for the gene disruption have a dramatic reduction in number and function of helper T cells. Reconstitution of these mice with a murine, wild-type CD4 transgene restores development of helper cells. Surprisingly, a mutant CD4 transgene whose product cannot associate with $p56^{lck}$ also partially restores selection of helper cells. CD4 is therefore required for positive selection of helper cells, but this function does not require its association with $p56^{lck}$. Mice lacking expression of CD4 were also able to delete thymocytes reactive with self superantigens, suggesting that CD4 signaling function may not always be essential for tolerance induction in the thymus.

This result contrasts with earlier studies in Dr. Littman's laboratory indicating that corecognition of MHC by a class I–specific TCR and CD8 was required for deletion of thymocytes in the presence of self antigen. Taken together, these results indicate that the requirement for CD4 and CD8 function is dependent on properties of individual TCRs.

Dr. Littman's laboratory earlier showed that the interaction of CD4 and $p56^{lck}$ is essential for the ability of T cell hybridomas to secrete interleukin-2 upon stimulation with antigen. To study functions of individual domains of $p56^{lck}$, chimeric molecules containing the external and transmembrane domains of CD4 were fused to the $p56^{lck}$ polypeptide and shown to reconstitute the response of the hybridoma to antigen. Other related cytoplasmic tyrosine kinases, including $p60^{c-src}$ and $p59^{fyn}$, were also able to function in the context of CD4 fusion proteins, indicating that these molecules can all interact with TCR-associated signaling components to potentiate T cell activation. Most surprising was the finding that kinase-negative mutants of the CD4-*lck* chimeric protein could also reconstitute the antigen-specific response in the T cell hybridoma. This result suggests that the CD4-associated $p56^{lck}$ molecule has a distinct kinase-independent function, possibly involving interaction with cytoskeleton or recruitment of signaling components into the TCR complex.

The developmental switch from double-positive to single-positive thymocytes involves the shut-off of either CD4 or CD8 transcription. The signal for gene down-regulation could be either instructed (through the TCR) or stochastic. Results in Dr. Littman's laboratory support a stochastic mechanism for the loss of expression of CD4. Details of this mechanism are poorly understood, but a pan–T cell transcriptional enhancer has been identified upstream from the CD4 promoter. It has recently been possible to demonstrate that, in transgenic mice, this enhancer directs appropriate developmentally regulated transcription from the CD4 promoter.

Dr. Littman and his colleagues have used this system to introduce an appropriately regulated human CD4 transgene into mice and have shown that development and function of helper T cells in CD4-negative mice are restored by expression of the transgene. Efforts are under way to identify sequences adjacent to the CD4 enhancer that control the shut-off of transgene transcription in $CD8^+$ cells.

A gene ablation approach has also been used to

prepare mice defective in expression of the lymphoid-specific CD2 glycoprotein. A substantial number of studies have suggested that the CD2 molecule, which interacts with several different ligands on antigen-presenting and target cells, has an essential role in T cell signal transduction and in lysis of target cells. The CD2-negative mice were found to be normal in all facets of immune system development and function, suggesting that the function of the highly conserved CD2 molecule may be redundant.

Mechanism of HIV Entry and Pathogenesis

HIV entry into target cells involves high-affinity binding of the viral envelope glycoprotein (gp120) to CD4, fusion of the viral membrane to cellular membranes, and uncoating of the virus. A number of observations indicate that viral binding to CD4 is not sufficient for its fusion to cellular plasma membranes. Retroviruses containing the HIV envelope glycoprotein and encoding selectable markers have been prepared as a tool for identifying additional factors involved in HIV entry. Only cells that bear these molecules as well as CD4 can internalize the virus and survive selection. This system is being used to complement genetic and biochemical approaches aimed at identifying additional host cell molecules required for HIV entry.

The selectable retrovirus system has also been used to study the effect of mutations in the envelope glycoproteins of HIV and of the related simian immunodeficiency virus (SIV). Mutations in the V3 loop of gp120 from HIV have been shown to influence virus-cell fusion after binding to CD4; analysis of mutations in this region argues that it is not a target of cellular proteases, as has been proposed by several groups. Growth of SIV in cultured human cells selects for rapidly replicating virus with a truncated envelope cytoplasmic domain. Using selectable HIV cores, Dr. Littman's group has shown that decreased infectivity of virus bearing full-length SIV envelopes is due to decreased envelope assembly onto particles and to lower fusogenic activity when compared with truncated envelopes.

The mechanism through which HIV infection results in loss of CD4$^+$ helper T cells is not yet understood. Since few CD4$^+$ cells are infected, it is likely that most of them die as a result of an indirect process. Mice in which helper T cell development and function are dependent on the human, rather than mouse, CD4 molecule have been developed, as described above. Although these animals cannot be infected with HIV, they are being used to test several hypotheses on the mechanism of HIV immunopath-ogenesis. For example, interaction of CD4 on helper cells with soluble or cell-bound envelope glycoprotein may sensitize these cells for autoimmune cytolysis or for the activation of a programmed cell death pathway. These mechanisms have been shown to occur *in vitro* and are now being tested *in vivo* using the murine model system developed in Dr. Littman's laboratory. (The projects described above on mechanisms of HIV entry were supported by a grant from the National Institutes of Health.)

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DEVELOPMENTAL BIOLOGY OF T LYMPHOCYTES

Dennis Y.-D. Loh, M.D., *Associate Investigator*

Thymus-derived lymphocytes (T cells) display three characteristic features. First, each T cell has a unique cell surface receptor, called the T cell receptor (TCR), by which antigens are recognized. Second, unlike the case of immunoglobulin molecules, antigens can only be recognized in the context of the products of the major histocompatibility complex (MHC), a phenomenon known as self MHC restriction. Third, T cells whose TCR specificity is directed against self antigens present in the organism must be rendered immunologically tolerant to avoid autoimmunity.

The focus of Dr. Loh's laboratory is the study of the developmental process by which functional T cells are produced. These studies involve the elucidation of the molecular mechanism of positive and negative selection of the thymocytes that forms part of the basis of modern T cell biology. Recent interest is centered on the development of transgenic mouse models to study the cells, molecules, and signals involved in determining T cell fate.

TCR Transgenic Mouse Whose Receptor Is MHC Class I Restricted

Previous work in Dr. Loh's laboratory, using 2C TCR transgenic mice, showed that thymocyte cell fate was determined by the interaction among the TCR, the co-receptor CD8 on the thymocyte surface, and the specific MHC class I molecules displayed in the thymus. Moreover, mutational analysis of the MHC molecule responsible for positive selection, K^b, showed that both the MHC-bound peptide and the amount of the available CD8 molecules were of crucial importance.

To fix the importance of the CD8 molecule, the 2C TCR transgenic mice were crossed with two kinds of CD8 transgenic mice. One overexpressed the CD8 constitutively, while the other had the CD8 gene deleted in the germline. In these double-transgenic mice, it was shown clearly that an excess of CD8 molecules on the thymocyte surface resulted in negative selection of the thymocytes (leading to programmed cell death), even in MHC backgrounds where positive selection (leading to cell survival and differentiation) was normally expected. On the other hand, no positive selection was observed when CD8 molecules were altogether absent in the $CD8^-/^-$ background. These results stress the importance of the CD8 as a co-receptor molecule that is crucial in determining cell fate.

Two distinct functions are possible for the CD8 molecule during selection. It can act as an adhesion molecule, binding to a molecule of MHC class I, or it can transmit an intracellular signal upon engagement. To decipher the relative contribution of each of these functions, Dr. Loh's laboratory has created a variety of CD8-mutant transgenic mice that are being backcrossed now to the $CD8^-/^-$ background. These studies should allow for the unequivocal determination of the mechanism by which CD8 determines thymocyte cell fate.

Transgenic TCR Mice Whose Receptor Is Directed Against a Specific Peptide Antigen

To study the role of specific peptides in determining thymocyte cell fate, Dr. Loh and his colleagues have utilized a TCR transgenic mouse whose receptor is directed against a specific peptide from chicken ovalbumin (cOVA). With this mouse model, they have shown that the *in vivo* administration of the peptide leads to negative selection of the developing thymocytes by a process called apoptosis or programmed cell death. Furthermore, to dissect the cellular and molecular requirements that lead to positive and negative selection, an *in vitro* model using the cOVA was developed.

Experimentally, transgenic thymocytes will undergo apoptosis in a developmentally stage-specific manner when the appropriate antigenic peptide is presented. Surprisingly, the results showed that any antigen-presenting cell was capable of initiating thymocyte death so long as it had the correct MHC class II molecule capable of binding the cOVA peptide. These results clearly indicate that the propensity for programmed cell death upon antigen contact is an inherent property of the immature thymocytes and is not determined by the nature of the antigen-presenting cells. In contrast, mature T cells proliferate upon antigen contact. Thus there is a clear-cut distinction of cell fate upon antigen contact between the immature thymocytes that undergo cell death and the mature cells that proliferate and live.

To start characterizing the variables that determine these diametrically opposite cell fates, Dr. Loh and his colleagues initially analyzed the dependence of antigen stimulation on tyrosine kinase activity. Using a combination of tyrosine kinase inhibitors, genistein and herbimycin A, they showed, in the *in vitro* selection system, that programmed cell death of the immature thymocytes appeared to be independent of the tyrosine kinase activity, while

the activation of the mature T cells could be totally blocked by the inhibitors. These results raise the intriguing possibility that the differential signaling apparatus used is stage-specific during thymocyte development.

How Is T Cell Tolerance to Extrathymic Antigens Produced and Maintained?

Clonal deletion appears to be the dominant mechanism by which self-reactive thymocytes are rendered inoperative if the self antigens are present in the thymus. Since most self antigens are derived from extrathymic tissues, the mechanism by which self-tolerance to such antigens is generated is an important one. To dissect this issue, Dr. Loh's group generated a transgenic mouse in which the MHC class I, L^d molecule is targeted exclusively to the exocrine pancreas. This mouse was then mated to the 2C TCR transgenic mouse, whose receptor specificity is against the L^d molecule.

Detailed analysis of the double-transgenic mice revealed no evidence of clonal deletion in the thymus. However, the peripheral T cells appeared to be anergic (unable to respond upon stimulation with the L^d molecule). Thus immunological tolerance appeared to be maintained, in this case, by the state of unresponsiveness rather than clonal deletion. Surprisingly, with the passage of time, the exocrine pancreas appeared to be undergoing inflammation by infiltrating lymphocytes in the double-transgenic mice.

These data strongly suggest that organ damage in the periphery may occur during the process of tolerization if the self-reactive thymocytes are created in the thymus and allowed to exit.

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CONTROL OF THE T CELL REPERTOIRE

Philippa Marrack, Ph.D., *Investigator*

In mice and humans the combinations of genetic elements, Vα, Vβ, Jα, and so on, that contribute to completed T cell receptors could create at least 10^{10} different ones. It is clear, however, that not all possible receptors are expressed in any given individual. The factors behind this—self tolerance, positive selection, and the genetic elements themselves—have been investigated.

Many T cells that could recognize self antigens die while developing in the thymus. The reasons for this are now being understood. Many antigens to self are expressed in the thymus, engaging the receptors on developing thymocytes with specificity for these antigens. Receptor ligation transduces signals—signals that include an increase in Ca^{2+} concentration within the cell. Unlike mature T cells, immature T cells die if their cytoplasmic Ca^{2+} levels rise above a certain point. Thus antigens expressed in the thymus screen out potentially autoreactive cells.

There are theoretical reasons to believe that not all self antigens reach the thymus. Recently Dr. Marrack showed this to be true. In collaboration

with Dr. John Freed, she isolated the peptides bound to class II major histocompatibilty complex (MHC) proteins on spleen and thymus. Although many were expressed in both tissues, some were present only in spleen. These peptides come from proteins expected to be at much higher concentration in the spleen than in the thymus because their expression is limited to B cells. Tolerance to this type of peptide is presumably not governed by deletion in the thymus, but rather by events in the periphery.

In the past Dr. Marrack's group and others demonstrated that mature T cells can become inactive if they contact peripheral antigens. This has been thought to be a result of exposure to massive doses of antigen that first stimulate the cells and then kill or inactivate them—a process called clonal exhaustion.

Experiments suggest, however, that this is not the only mechanism for induction of tolerance by peripheral antigens. In mice a retrovirus transmitted in milk, a mouse mammary tumor virus (MMTV), encodes a superantigen that reacts with most T cells bearing $V\beta14$. The mice are infected at birth, but subsequently the $V\beta14^+$ T cells disappear. Surprisingly, they disappear very slowly, with deletion complete when the mice are about 6 months old. There is no obvious indication that deletion is preceded by activation.

Although this is an interesting model for tolerance induction, it has its limitations. For example, little is known about the timing or location of expression of the viral superantigen. To deal with such problems, Dr. Marrack and her co-workers (partially supported by the National Institute of Allergy and Infectious Diseases, National Institutes of Health) have developed a different model. Adult mice are challenged every other day with minute doses of a bacterial superantigen, staphylococcal enterotoxin A (SEA). Chronic exposure to small amounts of SEA causes rapid and complete disappearance of mature target T cells, in this case bearing $V\beta3$.

Overall, these experiments show that tolerance can result from chronic exposure of mature T cells to small amounts of antigen. The work is leading to the suggestion that T cells are so programmed that they usually die or become inactive when they encounter antigen. It is only under unusual circumstances, when antigens are expressed in conjunction with some infectious agent, that T cells go into active response.

Positive selection remains an unsolved mystery. It involves contact between the receptor on developing thymocytes and MHC expressed on thymus cortical epithelial cells. Tolerance to MHC is also induced during thymocyte development, and it is not clear how self tolerance and positive selection can be reconciled. As mentioned above, thymocytes probably die when their receptors engage self because engagement induces high intracellular Ca^{2+} concentrations. However, Dr. Marrack's group has recently shown that when receptors are first expressed on thymocytes, they do not associate completely with CD3, the protein normally coexpressed with the receptor. Consequently, receptor engagement does not increase Ca^{2+} levels inside the cell. The group therefore suggested that positive selection may occur during this early stage of the thymocyte life history, at a time when receptor engagement cannot cause death.

It is clear that the receptor genes themselves affect the repertoire of T cell receptors. It is difficult to study such effects in the absence of other phenomena such as positive and negative selection. Since MMTV-associated superantigens have such a major effect on the T cell repertoire, a mouse strain of known MHC type and lacking all endogenous MMTVs is being developed for study. This is a lengthy process. Preliminary examination of several animals that are heterozygous for single MMTV integrants suggests that T cell use of $V\beta$s is very uneven. For example, $V\beta9$ is rarely used on T cells, and $V\beta2$ is used frequently. Similar findings apply in humans; for example, $V\beta10$ is used rarely and $V\beta13.1$ is used often. Such differences are probably due to the frequency with which the various $V\beta$ genes are rearranged.

By and large, the repertoire of receptors on CD4-bearing T cells is very similar on young and old animals. Surprisingly, this is not true for CD8-bearing T cells. As mice reach old age—18 months or more—spikes appear in the repertoires of their $CD8^+$ cells. For example, as many as 75% of the $CD8^+$ T cells in old mice may bear a single $V\beta$. The spiking cells do not appear to be leukemic. Currently the stimulating agent for these cells is being sought. Perhaps it is a tumor antigen.

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REGULATION OF B CELL DEVELOPMENT BY IMMUNOGLOBULIN M

MICHEL C. NUSSENZWEIG, M.D., PH.D., *Assistant Investigator*

Dr. Nussenzweig's research concerns the regulation of immunoglobulin genes and B lymphocyte differentiation. Areas currently under investigation include allelic exclusion, and the molecular basis for signal transduction by receptor immunoglobulins.

Dr. Nussenzweig and his colleagues, in experiments performed with transgenic mice, have established that allelic exclusion is regulated by the membrane-associated form of the immunoglobulin heavy chain. In these experiments, rearranged immunoglobulin genes were introduced into the germline of mice, and the membrane form of human IgM was found to inhibit the expression of endogenous mouse IgM. In contrast to membrane IgM, the secreted form of the same protein had no effect on endogenous immunoglobulin genes. These results raise two important questions that are currently the focus of Dr. Nussenzweig's laboratory: What happens at the genetic level that regulates exclusion? How does IgM transmit the message that ultimately results in exclusion?

Allelic Exclusion

There is persuasive evidence that exclusion is regulated at the level of immunoglobulin gene rearrangement. Recombination is believed to be initiated at both sets of parental loci and to be inhibited when one of the two alleles achieves a productive rearrangement. The finding that membrane immuno-

globulin is the feedback regulator for allelic exclusion has been confirmed by several groups. However, the mechanism that governs inhibition of endogenous immunoglobulin gene expression is not well defined.

One intriguing aspect of exclusion by transgenic immunoglobulin is that it is never complete. In order to characterize the molecular mechanisms responsible for exclusion in transgenic mice and to understand the basis for escape from regulation, Dr. Nussenzweig and his colleagues have examined the rearrangements of endogenous immunoglobulin gene segments. The results of these experiments suggested that the feedback signal generated by membrane-bound IgM produces a gradient along the immunoglobulin locus that prevents rearrangement of 5′ gene segments but has little effect on the 3′ elements. One way to account for these results would be to propose that exclusion is regulated by both the accessibility of a particular gene segment and the availability of the recombination machinery.

To test this two-step model for allelic exclusion and to explore the role of the recombinase-activating genes (RAG) in lymphocyte development, Dr. Nussenzweig and his colleagues have created transgenic mice that express the *RAG-1* and *RAG-2* genes in mature T cells. Neither gene alone has any effect. However, transgenic mice from six independently derived strains that carry both genes

develop a severe, progressive lymphoproliferative disorder, which results in death after 2–6 months. Early morbidity and mortality have made this disease difficult to study. However, Dr. Nussenzweig and his colleagues have made substantial progress in characterizing the pathology, and current efforts are focused on molecular etiology.

Gross examination shows enlarged spleens, lymph nodes, and lymphatic channels early in the disease. Moribund animals display a variety of phenotypes, including total body edema, enlarged kidneys and liver, and a number of different types of infections. Microscopic analysis reveals loss of architecture in lymph nodes and spleen, with a predominance of hystiocytoid cells. All other organs contain perivascular lymphoid infiltration, which only invades the parenchyma of the kidneys, liver, and lungs late in the disease.

Immunologic characterization shows increased numbers of lymphocytes in all classes, with a disproportionate increase in T cells of both CD4 and CD8 subclasses. Lymphocytes removed from spleens and lymph nodes proliferate spontaneously at a 20-fold greater rate than lymphocytes from wild-type litter mates. However, the proliferating cells are neither clonal (by Southern blotting analysis) nor malignant, since they do not form tumors in nude mice and do not grow continuously *in vitro*. Despite the abundance of lymphocytes, immune responses appear to be blunted severely in the RAG transgenic mice.

A number of molecular defects may explain this complex phenotype. One possibility is that deregulated expression of the RAG genes in mature T cells aborts allelic exclusion and results in continued T cell receptor (TCR) gene rearrangements. In this model, continued rearrangements would lead to formation of self-reactive T cell clones and a disease that is mediated by an autoimmune mechanism. A second hypothesis is that deregulated expression of RAG results in aberrant chromosomal translocations and abnormal expression of genes that regulate lymphocyte proliferation.

However, the phenotype may be entirely independent of recombination, since the RAG proteins are not known to be direct components of the recombination machinery. Indeed, the RAG products may function as gene activators, and aberrant gene activation may be another explanation for the transgenic phenotype. Dr. Nussenzweig and his colleagues are currently focusing on experiments that test these hypotheses, which should help to clarify the function of the RAG genes and further the understanding of allelic exclusion.

Signal Transduction by IgM

Membrane immunoglobulin is a key regulator in the B cell pathway. Early in B cell differentiation, IgM regulates allelic exclusion and B cell development. In mature B cells the same IgM protein functions as the antigen receptor that is responsible for detecting foreign antigens and triggering a cascade of events whose end result is specific antibody production. Despite the central role of receptor immunoglobulin, little is known about the mechanism by which this receptor produces a signal.

Two important features of the immunoglobulin antigen receptor have hindered understanding of its mechanism of signaling. First, its intracytoplasmic domain is composed of three amino acids that offer no specific clues about the mechanism of receptor function. Second, the receptor immunoglobulins are associated with several other polypeptides on the cell surface to form a multisubunit structure. Two of these receptor-associated polypeptides, MB-1 and B29, have been implicated in receptor assembly and cell surface transport. In addition, both are rapidly phosphorylated upon receptor crosslinking, but the functional role of the IgM-associated proteins is poorly defined.

As an initial step in studying the structural and functional requirements for signal transduction by immunoglobulin, Dr. Nussenzweig and his colleagues set out to reconstitute the immunoglobulin antigen receptor of B lymphocytes in the Jurkat T cell line by transfection of cloned components. T cell lines were chosen for these experiments for several reasons. First, there are no available models that faithfully reproduce the process of allelic exclusion *in vitro*. Second, T cells do not normally express either immunoglobulin or the IgM-associated proteins. Third, T cells are closely related to B cells and possess the specialized connections that are required for signal transduction by the TCR, which is highly homologous to immunoglobulins.

The reconstitution experiments showed that the combination of IgM and B29 was both necessary and sufficient to reconstitute antigen-specific signal transduction by immunoglobulin in the transfected T cells. IgM alone was not an active receptor in T cells. Crosslinking of the transfected IgM-B29 complex with either antireceptor antibodies or antigen induced cellular responses, such as calcium flux, phosphoinositol turnover, and interleukin-2 (IL-2) secretion. MB-1, a second IgM-associated polypeptide, was not required for either transport or signal transduction.

These experiments are the first to establish a requirement for B29 in immunoglobulin receptor

function. In addition, reconstitution of functional immunoglobulin antigen receptors in T cells offers a general solution to the difficult problem of major histocompatibility complex (MHC) restriction in transfer of cellular immunity. T cells that utilize immunoglobulin receptors would have the potential for recognizing any antigen that antibodies recognize in an MHC-independent fashion.

Dr. Nussenzweig is also Assistant Professor and Head of Laboratory at the Rockefeller University.

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NEUROTRANSMITTER RECEPTOR-MEDIATED RESPONSES IN LYMPHOCYTES

Donald G. Payan, M.D., *Assistant Investigator*

Dr. Payan and his colleagues have been studying neurotransmitter receptor modulation of lymphocyte surface molecules that regulate cell growth, differentiation, and cell-cell interactions. In addition, their research is focused on the biochemical characterization of the protease inhibitor domains of the molecule agrin. Agrin is a glycoprotein expressed in the brain and spinal cord during development and regenerative processes. It is also known to regulate the localization of acetylcholine receptors (AChRs) at the neuromuscular junction.

Substance P Receptor (SPR) Signal Transduction Mechanisms

A number of recent studies have shown that certain peptide receptors, when occupied by ligand, may alter their G protein subclass association according to the state of differentiation of the cell, resulting in diverse cellular responses. Given the wide spectrum of activities associated with SP (vasodilation, mitogenicity of cultured cells, neurotransmission, and change in the trophic and toxic cellular responses to amyloid β protein), Dr. Payan and his colleagues have focused their attention on the second messenger pathways that are activated following SPR stimulation.

The rat SPR cDNA was subcloned into the mammalian expression vector pRC/RSV and transfected into a number of cell lines. The group has isolated stable transfectants expressing the receptor and has demonstrated specific receptor message and ^{125}I-SP binding in SPR-transfected cells but not in untransfected cells or those transfected by vector alone.

The functional responses of the stable transfectants expressing the SPR were first examined by measuring SP-induced alterations in intracellular Ca^{2+} ($[Ca^{2+}]_i$), using the fluorescent indicator dye fura-2. Stimulation with SP transiently increased $[Ca^{2+}]_i$ in SPR-positive cells in a dose-dependent manner, with an $EC_{50} \sim 5 \times 10^{-10}$ M, a value similar to the K_d derived from ^{125}I-SP binding. To examine further the possibility that the SPR might also be coupled to an additional signaling pathway, intracellular cAMP levels following SP stimulation were quantified. The level of cAMP in SPR-positive cells was significantly increased following stimulation by SP and was maximal at 5–10 min. The effect of SP was dose-dependent, with an $EC_{50} \sim 5 \times 10^{-10}$ M, a value similar to that derived from the $[Ca^{2+}]_i$ mobilization studies. Dr. Payan's group has now been able to demonstrate, using forskolin and ionomycin, that in desensitization studies, the SPR signals these cells simultaneously and independently via the phosphatidylinositol (PI)-coupled and the cAMP-coupled pathways, and that the degree to which each pathway contributes to the overall SP-mediated response is determined by the relative abundance of different G proteins.

To examine whether SP stimulation results in the activation of downstream transcriptional regulatory factors, the group has transfected KNRK-SPR cells with plasmids containing the AP-1 and CRE (cAMP response element) enhancer elements coupled to the chloramphenicol acetyltransferase (CAT) reporter gene. Stimulation with SP over a concentration range of 1–1,000 nM results in a significant increase in CAT activity in both AP-1–CAT– and CRE-CAT–transfected KNRK-SPR cells. Northern and Western blot analyses demonstrate that the mechanism by which SP stimulates AP-1 enhancer

activity involves increases in both c-*jun* mRNA and protein. Moreover, gel retardation assays with oligomers containing the AP-1– and CRE-binding sites demonstrate that SP induces specific retardation bands consistent with increases in AP-1 and CRE complexes.

These experiments suggest that SP-mediated stimulation of cells involves the participation of two signaling pathways, resulting in the activation of several transcriptional regulatory mechanisms.

The Role of SP in Immune Responses: A Molecular Analysis

Previous work in Dr. Payan's laboratory, using well-defined animal models of inflammation and *in vitro* culture systems with mixed populations of lymphocytes, showed that SP amplifies and modulates certain aspects of the immune and inflammation responses. To analyze SP's "immunological properties" at the molecular level, the group has transfected the rat SPR cDNA into the Jurkat T lymphocyte cell line and has established stable transfectants. Jurkat-SPR cells demonstrate specific SP binding and functional responses to the peptide. Of great interest is the fact that in this cell line SP stimulation results in enhanced cell growth and the increased expression of interleukin-2 receptors and the cell-cell recognition molecule CD2.

Biochemical Characterization of Recombinant Rat Agrin

A full-length cDNA for rat agrin has been transiently expressed in COS cells and stably expressed in the neuronal-like cell line PC12. Dr. Payan's group is in the process of characterizing the recombinant agrin in terms of its AChR-clustering ability and also beginning to examine the biochemical properties of the protease-inhibitor domains present at the amino terminus of the molecule.

Using *in situ* hybridization with a probe that recognizes all agrin isoforms, Dr. Payan and his colleagues have demonstrated that agrin message is widely expressed during mammalian embryogenesis. In the developing rat, particularly high levels of expression are found in the dorsal root and cranial ganglia, gut, whisker rudiments, penis, snout, teeth, retina, hippocampus, cerebral cortex, and the lining of brain ventricles. Functional analysis of the recombinant rat protein shows that it is a potent inhibitor of the proteases trypsin, chymotrypsin, and plasmin, but not thrombin or the plasminogen activators. Dr. Payan and his colleagues conclude that agrin may play multiple roles in mammalian development, including the regulation of proteolysis in the extracellular matrix.

Dr. Payan is also Associate Professor in residence in the Departments of Medicine (Infectious Disease) and Microbiology and Immunology at the University of California, San Francisco.

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SIGNAL TRANSDUCTION IN HEMATOPOIETIC CELLS

Roger M. Perlmutter, M.D. Ph.D., *Investigator*

Although substantial progress has been made in defining the cell surface molecules responsible for specific recognition of antigens by B and T lymphocytes, the signal transduction pathways that couple ligand binding by these receptors to changes in cell behavior remain largely enigmatic. Members of Dr. Perlmutter's laboratory have employed molecular genetic strategies to identify important constituents of these signaling pathways and to manipulate lymphocyte signaling experimentally *in vivo* through the generation of transgenic mice.

G_i Proteins Regulate Lymphocyte Migration

Guanine nucleotide–binding (G) proteins are components of many signal transduction pathways in nonlymphoid cells and have previously been implicated in signaling from the T cell antigen receptor. Members of Dr. Perlmutter's laboratory have employed a novel strategy to investigate the importance of one class of G proteins (the G_i proteins) in T cell signaling. These experiments take advantage of the fact that G_i proteins can be specifically and irreversibly inactivated by pertussis toxin–mediated ADP-ribosylation. Transgenic animals were generated in which essentially all thymocytes express the catalytic subunit of this toxin under the control of a T cell–specific promoter derived from the *lck* gene.

The pertussis toxin–bearing thymocytes exhibit no defects in T cell receptor–mediated stimulation, but instead manifest a remarkable inability to colonize peripheral lymphoid tissues correctly. These experiments demonstrate that a pertussis toxin–sensitive process, almost certainly involving a G_i protein, assists in regulating lymphocyte trafficking. Detailed studies have excluded most of the known cell adhesion phenomena as potential targets of pertussis toxin. Hence current experiments focus on G_i protein–mediated chemotaxis to explain the migratory behavior of normal lymphocytes. More recent experiments indicate that the earliest migration events, which establish the organization of cortical and medullary elements in the thymus, also proceed via a pertussis toxin–sensitive process. Current efforts are focused on the receptor and effector mechanisms that regulate the migratory behavior of immature thymocytes.

Control of Thymocyte Signaling by Protein-Tyrosine Kinases

Members of Dr. Perlmutter's laboratory have adduced persuasive evidence positioning two membrane-associated protein-tyrosine kinases, the products of the *lck* and *fyn* genes, in the signaling pathway that is activated by ligand occupancy of the T cell antigen receptor. The *lck* gene product, $p56^{lck}$, is expressed in a lymphocyte-specific fashion and was previously shown to interact with the CD4 and CD8 co-receptor components of the T cell antigen receptor complex. A comprehensive series of experiments performed in transgenic mice has provided evidence linking $p56^{lck}$ activity to the control of thymocyte maturation. Moreover, $p56^{lck}$ also interacts with the signaling component of the interleukin-2 receptor. Hence this kinase participates in the regulation of multiple T cell signaling pathways.

One consequence of overexpression of $p56^{lck}$ in otherwise normal thymocytes is the rapid development of thymic tumors. Indeed, several lines of animals in which tumors reproducibly develop in the first 8 weeks of life have now been propagated for more than 10 generations. Tumorigenesis occurs as a consequence of the amount of $p56^{lck}$ activity present in individual cells. Introduction of an "activating" mutation (which eliminates a carboxyl-terminal phosphorylation site) into the *lck*-coding sequence produces an even more potent oncogene. The tumors that arise in *lck* transgenic mice resemble lymphoblasts in some cases of human acute lymphocytic leukemia, where translocation of the *lck* gene has been observed. Although the frequency of such activation events is unknown, *lck* transgenic mice provide a valuable test system for the study of novel therapies for at least some forms of human malignant disease.

A second protein-tyrosine kinase, $p59^{fynT}$, is also expressed exclusively in hematopoietic cells and appears to transmit activating signals directly from the T cell antigen receptor. Transgenic mice in which augmented lymphocyte-specific expression of $p59^{fyn}$ has been achieved contain thymocytes that respond too vigorously to ordinary stimuli. This observation permitted application of an especially powerful strategy to the analysis of $p59^{fyn}$. In this case a mutation was introduced into the *fyn* transgene that abrogated catalytic activity, producing a "dead" kinase. Overexpression of this inactive mutant protein provoked a dramatic reduction in the response of otherwise normal thymocytes to T cell receptor–specific stimuli. This result almost certainly reflects competition between active and inactive forms of $p59^{fyn}$ for appropriate cellular targets (either receptor structures or substrates).

In more recent experiments it has been possible to use homologous recombination in embryonic stem cells to produce mice bearing a homozygous disruption of the *fyn* locus. Detailed analysis of these animals promises to permit a more complete dissection of the biochemical wiring that links lymphocyte surface proteins to regulators of gene expression.

The *lck* Promoter System

Because p56*lck* participates in the control of normal thymocyte development, it is perhaps not surprising that the expression of the *lck* gene is itself tightly regulated. Members of Dr. Perlmutter's laboratory have characterized the structures of the murine and human *lck* genes and have defined two promoters in each gene that function in a developmentally restricted fashion. The 3′ (or proximal) *lck* promoter is active only in immature thymocytes, while the 5′ (or distal) promoter functions mainly in mature T cells. Through a series of gene deletion and mapping experiments, minimal sequences capable of directing tissue-specific and temporally correct expression of heterologous DNA elements have been defined for both promoters.

In this way, an extremely useful set of reagents has been developed for use in the generation of transgenic mice. Indeed, more than 30 different genes have been expressed in a thymus-specific fashion in transgenic mice using the proximal *lck* promoter. It now appears that expression of *lck* transcripts marks an important early step in T cell lineage commitment. A thorough dissection of the nuclear factors that regulate *lck* gene expression promises to illuminate mechanisms controlling thymopoiesis.

Dr. Perlmutter is also Professor of Immunology, Medicine, and Biochemistry at the University of Washington School of Medicine, Seattle.

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REGULATION OF EXPRESSION OF CLASS II MHC AND HIV GENES

B. Matija Peterlin, M.D., *Associate Investigator*

Dr. Peterlin and his co-workers are studying the regulation of expression of class II major histocompatibility complex (MHC; class II, DR) and human immunodeficiency virus (HIV) proteins. In humans, different levels of expression of these proteins lead to severe combined immunodeficiency and/or autoimmunity. Class II and HIV are further linked by genetics, since low expression of each can be complemented in trans, i.e., by rescuing the gene(s) missing or mutated in the class II bare lymphocyte syndrome (BLS II; one of only two known hereditary deficiencies in a regulatory protein in

humans) and by HIV-encoded trans-activators Tat and Rev.

Regulation of Class II Gene Expression

Class II antigens are peptide carriers that present self and foreign peptides on antigen-presenting cells to T cells to initiate, and to B cells to propagate, the immune response. Developmentally, class II antigens must be expressed in the thymus to tolerize T cells to self peptides and to restrict T cells to nonself peptides. For these interactions to occur, the

expression of class II genes must be carefully regulated.

To dissect this complex regulation, Dr. Peterlin first analyzed cis-acting sequences in a number of class II genes. This resulted in functional delineation of transcriptional enhancer elements and conserved upstream and downstream promoter sequences. Class II transcriptional enhancers are lymphoid specific. Upstream promoter elements are responsible for B cell–specific and interferon-γ (IFN-γ)-inducible expression, while downstream promoter elements position the site of initiation of DRA transcription. Identical cis-acting sequences are responsible for B cell–specific and IFN-γ–inducible regulation. By creating a cassette-forming synthetic DRA promoter, Dr. Peterlin was able to multiply certain conserved elements and to exchange promoter elements between class II genes. Subtle differences in transcriptional regulation of DR, DP, and DQ genes, as well as defects in the expression of DOB, DQA2, and DQB2 genes, were revealed.

Next, Dr. Peterlin turned his attention to trans-acting factors that interact with conserved upstream promoter sequences. These are composed of reiterated motifs. For example, consensus activator protein-1 (AP-1) and cAMP-responsive element (CRE) binding sites flank those for helix-loop-helix (HLH) proteins—regulatory factor-X (RF-X) and Ephrussi factors 12 and 47 (E12/E47). Not only are these proteins modified post-transcriptionally, but they interact with one another to direct tissue-specific and IFN-γ–inducible regulation of class II genes. Moreover, RF-X is translated from two different methionine codons. Functions of full-length and truncated RF-X are under investigation.

Other aspects of this complex transcriptional regulation were revealed. For example, AP-1, which is present in cells that do not express class II determinants, negatively regulates class II transcription. In B cells, c-Jun does not form heterodimers with c-Fos, and AP-1 does not bind to DNA. However, following B cell activation, c-Fos forms heterodimers with CRE-BP1, and this new heterodimer increases levels of class II transcription. In contrast, E12/E47 and RF-X are positive regulators in B cells. Moreover, E12/E47 might regulate the developmental expression of class II genes.

Finally, Dr. Peterlin studies genetic defects in BLS II where transcriptional regulators are congenitally absent. Minimal class II promoter elements and isolated trans-acting factors have facilitated these analyses. First, interactions between E12/E47 and RF-X are nonfunctional in BLS II cells. Second, since both of these proteins are present, either post-transcriptional processes or their coactivators are mutated or missing in this disease. Using biochemical techniques and genetic approaches, Dr. Peterlin hopes to characterize these defective proteins and to isolate their gene(s). (The project described above is supported by a grant from the National Institutes of Health.)

Regulation of HIV Gene Expression

HIV, the cause of acquired immune deficiency syndrome (AIDS), requires the expression of trans-activators Tat and Rev for efficient viral replication and gene expression. Dr. Peterlin has been studying steps leading to the activation of HIV transcription and the mechanism of action of the virally encoded Tat protein.

HIV transcription is initiated by the release of nuclear factor κB (NF-κB) and of nuclear factor of activated T cells (NFAT) from the cytoplasm to the nucleus of infected cells. These trans-acting factors interact with HIV enhancer sequences and result in increased loading of RNA polymerase II. For the activation of transcription, p50, which is the DNA-binding subunit of NF-κB, interacts with p65. Recently Dr. Peterlin observed that the tyrosine kinase pathway plays an important role in the cytoplasmic-to-nuclear translocation of NF-κB. In particular, in T cells that lack a receptor tyrosine phosphatase (CD45), NF-κB is constitutively active in the nucleus.

However, although increased rates of initiation of HIV transcription are observed with NF-κB and NFAT, assembled transcription complexes do not elongate efficiently through viral coding sequences. For that, Tat has to interact with its trans-acting responsive region (TAR). Tat binds to the TAR RNA stem-loop with the help of cellular RNA-binding proteins. In rodent cells, where lower levels of trans-activation are observed, these cellular RNA-binding proteins are missing.

Further evidence for these conclusions came from experiments where Tat was fused to heterologous RNA-binding proteins—for example, the coat protein of bacteriophage MS2 and the RNA-binding domain of Tat of the equine infectious anemia virus (EIAV). TAR was replaced by appropriate RNA targets, the MS2 operator and TAR of EIAV. These chimeric Tats functioned equivalently in human and rodent cells.

Hybrid trans-activators also led to extensive mapping of activation and Tat RNA-binding domains of HIV-1, HIV-2, and EIAV. In addition, a minimal lentiviral TAT of only 25 amino acids was constructed, where 15 amino-terminal and 10 carboxyl-terminal residues represent activation and RNA-binding domains, respectively. Structural studies of this mini-

mal Tat are in progress. Dr. Peterlin is now using genetic and biochemical approaches to isolate, characterize, and express proteins that interact with the Tat activation domain.

Dr. Peterlin is also Associate Professor of Medicine and of Microbiology and Immunology at the University of California, San Francisco.

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GENERATING A REPERTOIRE OF ANTIGEN-SPECIFIC RECEPTORS DURING DEVELOPMENT OF THE IMMUNE SYSTEM

DAVID G. SCHATZ, PH.D., *Assistant Investigator*

Recognition of infectious agents and other nonself antigens by the immune system depends on antigen receptor molecules (immunoglobulins and T cell receptors) expressed on the surface of B and T lymphocytes. Each immunoglobulin and T cell receptor molecule is highly specific for a given antigen, so the immune system requires a wide array of different receptors to cope with the diversity of potential antigens in the environment. The myriad different genes needed to encode these receptors are assembled from component gene segments by a site-specific recombination process known as V(D)J recombination (so named for the V [variable], D [diversity], and J [joining] gene segments used in the reaction). V(D)J recombination, unique and essential to the development of B and T lymphocytes, is the only truly site-specific recombination process known in vertebrates. Dr. Schatz's laboratory is interested in answering two fundamental questions concerning V(D)J recombination: What is the biochemical mechanism of the reaction? What molecular mechanisms regulate the reaction during lymphoid development?

This recombination reaction has been intensively studied since its discovery in 1976, yet little has been learned about the enzymatic machinery (recombinase) that carries it out. Particularly frustrating had been the inability to identify the gene or genes encoding the V(D)J recombinase, despite a detailed understanding of the substrates and products of the reaction. Dr. Schatz, while working in Dr. David Baltimore's laboratory, developed a novel, genetic approach for the identification and isolation of such genes. It combined a sensitive assay for V(D)J recombinase activity with a standard gene transfer technique (genomic transfection). This approach allowed Drs. Schatz, Marjorie Oettinger, and Baltimore to identify a genomic locus that could activate the V(D)J recombinase when transferred into nonlymphoid cells, and subsequently to demonstrate that two genes within that locus were necessary and sufficient for this activity. These two recombination-activating genes (*RAG-1* and *RAG-2*) have become the prime candidates for the genes that encode the critical, lymphoid-specific components of the V(D)J recombinase.

In their further characterization of *RAG-1* and *RAG-2*, Dr. Schatz and his colleagues demonstrated that the two genes are coexpressed only in developing lymphocytes—in exactly those cells that are assembling immunoglobulin and T cell receptor genes. In collaboration with Dr. Jerold Chun, how-

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ever, they also found that *RAG-1*, but apparently not *RAG-2*, is transcribed in neurons in the central nervous system, raising the possibility that *RAG-1* has a role in processes other than classic V(D)J recombination. Dr. Schatz's laboratory is currently testing the hypothesis that *RAG-1* is involved in recombination in the central nervous system by studying the behavior of recombination substrates in neuronal cell lines and by characterizing circular DNA molecules (thought to be the by-product of recombination) isolated from the central nervous system.

Because of their intimate connection with V(D)J recombination, *RAG-1* and *RAG-2* provide important tools for the study of the molecular mechanisms regulating the recombination process. Using them as indicators, Dr. Schatz and his colleagues are studying when and how the V(D)J recombinase is turned on and then off again as B and T cells develop. Initial studies, done in collaboration with Dr. Craig B. Thompson (HHMI, University of Michigan) and his colleagues, demonstrated that signals transduced through surface T cell receptor molecules are capable of down-regulating expression of RAG-1 and RAG-2 in thymocytes, a result that has helped establish a new paradigm for how expression of the V(D)J recombinase is terminated.

V(D)J recombination is regulated not only at the level of expression of the recombinase, but also at the level of the availability, or "accessibility," of the individual immunoglobulin and T cell receptor gene segments. Dr. Schatz and his colleagues have demonstrated that nonlymphoid cells expressing high levels of RAG-1 and RAG-2 do not recombine their endogenous immunoglobulin or T cell receptor gene segments, despite their high levels of recombinase activity. The laboratory is currently determining whether the introduction of cloned transcription factors, known to bind to the enhancers and promoters of immunoglobulin and T

cell receptor genes, into such nonlymphoid cells can activate the rearrangement of immunoglobulin and T cell receptor gene segments. Such reconstruction experiments could provide a more detailed understanding of how DNA structure affects the V(D)J recombination process.

Finally, a central goal of Dr. Schatz's laboratory is to understand the enzymatic mechanism of V(D)J recombination, an elusive goal thus far, largely because efforts to reconstitute the reaction in a cell-free system have been unsuccessful. As a first step toward this goal, the group is working to obtain highly purified preparations of the RAG-1 and RAG-2 proteins, and antibodies that specifically recognize these proteins. With these tools, the laboratory will examine the biochemical properties of the RAG proteins, asking in particular whether they exhibit the activities expected of proteins involved in recombination (e.g., topoisomerase, endonuclease, exonuclease, or ligase activities) and whether they bind DNA in a sequence-specific manner. Also to be addressed are questions concerning the cellular and subcellular localization of the RAG proteins and the identity of other proteins, if any, with which they interact. (This aspect of the laboratory's work was supported by a grant from the National Institutes of Health.)

Dr. Schatz is also Assistant Professor of Immunobiology at Yale University School of Medicine.

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REGULATED AND BASAL TRANSCRIPTION OF LYMPHOCYTE-SPECIFIC GENES

STEPHEN T. SMALE, PH.D., *Assistant Investigator*

Mature B and T lymphocytes arise from hematopoietic stem cells through a complex but highly ordered sequence of events. A central component of this developmental process is the precise activation and inactivation of a large pool of genes expressed with a wide variety of temporal and cell-specific patterns.

Dr. Smale's laboratory examines the mechanisms

responsible for gene regulation in developing lymphocytes. These studies focus on two distinct areas. First, an analysis of the terminal deoxynucleotidyltransferase (TdT) gene is directed toward identifying transcription factors responsible for restricting gene expression to lymphoid cells. Second, studies of core promoter regions found in lymphocyte-specific genes are conducted to elucidate general

mechanisms used to direct transcription initiation by RNA polymerase II.

Lymphocyte Specificity of TdT Transcription

Dr. Smale and his colleagues have focused on the TdT gene for their studies of transcriptional regulation because of its expression patterns in normal and leukemic cells. TdT is a template-independent DNA polymerase that inserts nucleotides at the D-J (D, diversity; J, joining) and V-DJ (V, variable) junctions during immunoglobulin and T cell receptor gene rearrangements. In normal cells, TdT expression is unusual because it is found in both early B and early T cells, suggesting that it may be regulated by transcription factors common to these two related but distinct lineages. Moreover, TdT is expressed at high levels in acute lymphocytic leukemias (ALLs) and at lower levels in a subset of acute myelocytic leukemias (AMLs). TdT expression in AMLs is particularly intriguing because it suggests that the regulatory protein(s) that deregulates TdT expression (the TdT gene is not transcribed in normal myeloid cells) may also play a role in leukemogenesis.

A major objective of Dr. Smale's laboratory has been to understand the important protein-DNA interactions that occur at a DNA sequence element called D', which is located ~60 bp upstream from the TdT transcription start site. This element is essential for TdT promoter activity in lymphoid cell lines and is capable of interacting with multiple DNA-binding proteins. Previously Dr. Smale identified and purified a protein called LyF-1, which interacts with both the D' element and a second element in the TdT promoter and is expressed at high levels in lymphoid cells. LyF-1 is also bound to the promoters for several other lymphocyte-specific genes, suggesting that it may be a transcriptional activator for a variety of genes expressed specifically in the B and/or T lymphocyte lineages.

Dr. Smale's laboratory has now demonstrated that in addition to LyF-1, members of the ets family of nuclear oncoproteins bind specifically to the D' region and overlap the LyF-1–binding sites. Some of the ets members, including ets-1 and fli-1, are expressed predominantly in lymphoid tissues. A detailed characterization suggests that two molecules of LyF-1 bind immediately adjacent to each other on the D' element and that the ets-binding site directly overlaps the more proximal of the two LyF-1 sites.

Analysis of a series of substitution mutations suggests that both LyF-1 and an ets protein may be essential for TdT transcriptional activation. Lending further support to this hypothesis is the finding that the binding sites for LyF-1 and ets proteins are conserved through evolution, as both proteins bind to the human TdT promoter as well as to the murine promoter. Further studies are directed toward analyzing the interplay between these two proteins and determining which proteins might be responsible for TdT deregulation in acute myelocytic leukemias. (This work was supported by a grant from the National Institutes of Health.)

Transcription Initiation from Promoters That Lack TATA Elements

A TATA box is a common control element found in the promoters for most genes that have been identified and in virtually every promoter that has been analyzed in biochemical detail. This element is not present in the TdT promoter, nor in the promoters for many other lymphocyte-specific genes (e.g., λ5, VpreB, *lck*, *B29*, and pp52). To gain further understanding of transcription initiation in the absence of a TATA box, Dr. Smale's laboratory has been analyzing a core element in the TdT promoter that appears to carry out the same functions as TATA. This element, called an initiator (or Inr), is distinct from TATA in that it overlaps the transcription start site rather than, like TATA, being located 30 bp upstream. Previous studies by Dr. Smale demonstrated that the Inr acts in concert with upstream activator elements, in the absence of TATA, to direct high levels of accurately initiated transcription. Moreover, Dr. Smale and others surprisingly have found that transcription from promoters lacking a TATA box and containing an Inr element is absolutely dependent on the TATA-binding protein (TBP).

A number of studies in the laboratory are directed toward further understanding of the biological and biochemical characteristics of Inr elements. One of these studies addresses the role of TBP during transcription from TATA-less promoters. The nucleotide sequence 30 bp upstream from the TdT Inr was varied extensively, and each resulting promoter was tested for transcriptional activity and for TBP binding. Efficient Inr-mediated transcription was found with most promoters, but promoter strength was greatly reduced by highly GC-rich sequences that possess the lowest affinity for TBP.

These results suggest that TBP must interact with the −30 region during transcription initiation mediated by an Inr and that most, but not all, sequences interact with an affinity sufficient for high transcription levels. Interestingly, the −30 region of the TdT promoter contains a sequence that is highly GC-rich and relatively poor in promoting Inr-mediated transcription. Future studies will determine whether

this characteristic is an important feature of the TdT promoter and whether it contributes to appropriate transcriptional regulation in developing lymphocytes.

Dr. Smale is also Assistant Professor of Microbiology and Immunology and a member of the Molecular Biology Institute at the University of California, Los Angeles, School of Medicine.

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CONTROL OF CELLULAR REGULATION BY PROTEIN-TYROSINE PHOSPHATASES

MATTHEW L. THOMAS, PH.D., *Assistant Investigator*

Dr. Thomas and his colleagues are interested in defining the interactions and functions of protein-tyrosine phosphatases (PTPases) expressed by cells of the immune system. Studies in recent years from this and several other laboratories have demonstrated that PTPases are a large, diverse group of enzymes critical in the regulation of the cell cycle, differentiation, and activation. The Thomas group's research efforts are focused on determining the function, characterization, and expression of individual PTPases.

Characterization of Hematopoietic Intracellular PTPases

Lymphocyte activation induced by engagement of either the T cell antigen receptor or membrane immunoglobulin results in a rapid change in tyrosine phosphorylation. The exact kinases and phosphatases responsible for regulating antigen-induced activation are not known, although members of the Src-family tyrosine kinases and the transmembrane PTPase CD45 have all been implicated. Defining the coordinate interaction between the kinases and phosphatases in regulating cellular functions is emphasized in the Thomas group's research activities.

Initial studies characterized the hematopoietic-specific CD45 PTPase. Subsequently a large number of cDNAs with significant sequence similarity to CD45 were identified from an invertebrate species, *Styela plicata*. This indicated that the PTPase family

is extensive and provided the basis for identifying additional PTPases expressed by hematopoietic cells.

Screens of mouse tissues and cell lines revealed several novel phosphatases, a limited number of which were primarily expressed by cells of the immune system. One of these PTPases, SHP, was predicted to be intracellular. Development of a specific antiserum allowed the demonstration that SHP is a cytosolic protein. SHP is expressed by several distinct lineages of leukocytes, including macrophages, and by T and B lymphocytes. The pattern of SHP expression within tissues was examined by *in situ* hybridization, using a complementary RNA-derived probe. SHP is expressed widely in lymphoid tissues, but more abundantly in the medulla of the thymus, with its more mature thymocytes, and in follicles of spleen and lymph nodes, areas rich in B cells.

The structure of SHP predicts that it may be directly involved in regulating signal transduction. The amino-terminal domain contains two tandem SH2 domains. SH2 domains have been demonstrated to bind phosphorylated tyrosine or serine residues, thus providing a means by which phosphorylation results in interactions with other proteins. It is likely that there are specific interactions for each SH2 domain. Dr. Thomas's group expressed bacterial fusion proteins containing SHP SH2 domains and demonstrated specific interactions with a subset

of phosphotyrosine proteins from the thymoma cell line LSTRA. Thus SHP may interact through the SH2 domains with a specific set of tyrosine-phosphorylated proteins.

PEP is an additional intracellular PTPase expressed by many different types of leukocytes. In contrast to SHP, it is revealed by *in situ* hybridization to be uniformly expressed in thymus, spleen, and lymph nodes. Sequence analysis of PEP disclosed several putative nuclear-localizing signals. Furthermore, near the carboxyl terminus there are sequence motifs indicative of rapid degradation, suggesting that PEP protein expression may be highly regulated.

Phosphorylation is an important regulator of transcriptional activity, yet tyrosine phosphorylation's role in regulating transcription is unknown. To analyze whether PEP is a nuclear phosphatase, members of Dr. Thomas's laboratory epitope-tagged PEP by the addition of a small sequence with a defined antibody-binding site. Transfection of the epitope-tagged PEP into HeLa cells revealed a nuclear localization. This raises the exciting possibility that PEP may function in regulating nuclear events. Further insights into the role of tyrosine phosphorylation in controlling nuclear events should follow from mutagenesis dissection of PEP function.

Transmembrane PTPases Expressed by Lymphocytes

CD45 is an abundant transmembrane PTPase of leukocytes. Dr. Thomas and his colleagues functionally characterized CD45 by developing CD45-deficient T cell lines. This research demonstrated a critical role for CD45 in antigen-induced activation. CD45 deficiency leads to the inability to respond properly to T cell receptor stimuli. Thus signaling through the T cell receptor requires the expression of CD45. However, it is possible to bypass the signaling defect by directly activating protein kinase C through T cell receptor stimulation. This suggests that CD45 functions proximal to protein kinase C activation.

Analysis of tyrosine-phosphorylated proteins in the CD45-deficient cells revealed that members of the Src–tyrosine kinase family are increased in tyrosine phosphorylation and are therefore potential substrates for CD45. Src-family members are negatively regulated by tyrosine phosphorylation of a carboxyl-terminal site. Thus the increase in phosphorylation in Src-family members in CD45-deficient cells may lead to decreased kinase activity.

Three members of the Src family are expressed in T cells, $p56^{lck}$, $p59^{fyn}$, and $p62^{yes}$. Examination by members of Dr. Thomas's laboratory has indicated that the kinase activities for all three are decreased in the CD45-deficient T cells. Furthermore, in collaboration with Dr. Bart Sefton of the Salk Institute, analysis of the negative regulatory site demonstrated increased phosphorylation at this site in the CD45-deficient cells. This indicates that CD45 functions to regulate Src-family-member kinase activity by dephosphorylating the negative regulatory site. For T cells, this function is required for signaling through the T cell receptor. (Work on CD45-deficient cells is supported by grants from the National Institutes of Health and the Council for Tobacco Research.)

T cells also express an additional transmembrane PTPase, LRP. LRP was initially identified by Dr. Thomas's group by its similarity to CD45. cDNA sequence analysis predicts a highly glycosylated exterior domain of 123 amino acids. Dr. Thomas and his colleagues developed a monoclonal antibody that recognizes the exterior domain of LRP. Use of this antibody to immunoprecipitate biosynthetically labeled LRP confirms that the protein is post-translationally modified by *N*- and *O*-linked glycosylation. Furthermore, staining of cells with anti-LRP indicates that LRP is expressed at low levels on many cell types, including lymphocytes. Interestingly, CD45-deficient cells express LRP, indicating that LRP does not compensate for loss of CD45 function. The anti-LRP antibody will facilitate the analysis of LRP function.

Dr. Thomas is also Associate Professor of Pathology and Assistant Professor of Molecular Microbiology at Washington University School of Medicine, St. Louis.

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MOLECULAR ASPECTS OF DIFFERENTIATION AND PROLIFERATION OF THE LYMPHOID SYSTEM

CRAIG B. THOMPSON, M.D., *Associate Investigator*

Dr. Thompson and his colleagues are focusing on the characterization of molecular events associated with cellular differentiation and proliferation of the immune system. At present the laboratory is mainly engaged in investigating 1) the regulation of gene expression during human T cell activation and proliferation and 2) B cell development in the avian bursa of Fabricius.

Normal Human T Cells as a Model System for Study of Gene Expression During Cellular Activation

For a number of years the laboratory has studied the expression and function of nuclear oncogenes. Since many of the nuclear proto-oncogenes play important roles in the regulation of cellular proliferation, this work has led to an expanded interest in understanding molecular events associated with the transition of a cell from a quiescent state to one of either cellular proliferation or effector function. To study these events in a normal cell population, the group has chosen to study molecular events associated with human T cell activation.

The laboratory has concentrated on how molecular events transduced through the T cell receptor and accessory T cell surface molecules regulate the expression of genes associated with cellular proliferation and T cell function. These genes include the nuclear proto-oncogenes, lymphokine genes, T cell receptor genes, and activational T cell surface receptors. Over the past several years, the group has been concentrating on the expression and function of the *Ets* family of proto-oncogenes, and has been able to demonstrate that these genes are reciprocally expressed during T cell activation. *Ets*-1 is selectively expressed in quiescent cells, while *Ets*-2 is expressed in cells activated through the T cell receptor. Recent studies in collaboration with Dr.

Jeffrey Leiden (HHMI, University of Michigan) have demonstrated that *Ets*-1 plays a major role in the transcriptional regulation of T cell quiescence. In contrast, *Ets*-2 may play a role in the transcriptional induction of T cell activation genes required for cell proliferation.

In addition, Dr. Thompson's group has now identified several additional *Ets*-related genes that are differentially expressed during T cell activation. One of these genes, *Elf-1*, appears to play an important role in the regulation of HIV-2 (human immunodeficiency virus type 2) as well as several T cell activation genes. Surprisingly, the Elf-1 protein was found to be expressed constitutively in the T cell nucleus.

To explain how Elf-1 could play a role in activational gene expression, the group has been examining the molecular interactions of Elf-1 with other nuclear regulatory proteins. In resting T cells, Elf-1 appears to bind specifically to the retinoblastoma protein Rb. As T cells undergo activation and cell cycle progression, Rb is phosphorylated, and phosphorylated Rb does not bind to Elf-1. Thus Elf-1 is a transcription factor that directly interacts with a protein involved in cell cycle regulation. Studies are under way to elucidate how these interactions regulate Elf-1 transcriptional events.

The laboratory has also continued to characterize a novel T cell activation pathway that is both defined and regulated by the CD28 surface molecule. The CD28 pathway coordinately regulates the expression of lymphokine genes in antigen-activated T cells. Lymphokine production is one of the major effector functions of T cells and serves to regulate the responses of many of the other types of inflammatory cells. Several new aspects of signal transduction through CD28 have been defined.

CD28 activation involves specific induction of a

tyrosine kinase, and activation of the kinase is required for all subsequent events associated with CD28 activation. However, immunoprecipitation of CD28 from activated T cells has failed to coprecipitate a tyrosine kinase but has allowed the identification of a novel low-molecular-weight protein that specifically associates with CD28. Work to identify the mechanism of tyrosine kinase activation by CD28 is under way.

The natural ligand for CD28, B7, is expressed on activated antigen-presenting cells. In collaboration with Dr. Peter Linsley, Dr. Thompson's group has succeeded in demonstrating that B7-dependent T cell activation is required for the initiation of allograft rejection *in vivo*. These studies, and those of several other groups, demonstrate the importance of CD28 activation in the initiation of some forms of immune response. Studies are under way to determine whether additional co-stimulatory pathways exist and, if so, what role they play in regulating the subsequent immune response.

The Bursa of Fabricius as a Model System for Study of Lymphoid Development

The avian bursa of Fabricius provides a unique organ for the study of lineage-specific development in a multicellular organism. In the chicken, B cells develop in a single wave, beginning with commitment of progenitor cells to B cell differentiation between days 10–15 of embryogenesis. By day 18 all lymphoid progenitor cells capable of B cell differentiation have migrated to the bursa of Fabricius. Once these cells enter the bursa, they begin to grow exponentially and to populate the bursal follicles. Between day 18 of embryogenesis and 4 weeks of age, B cells undergo a stage of bursal-dependent differentiation, and by the end of this period, chickens are able to mount primary immune responses against virtually all antigens. At 4 weeks of age, sufficient numbers of B cells have migrated from the bursa to peripheral lymphoid organs so that even if the bird is bursectomized, the B cell immune system is maintained.

Over the past several years, Dr. Thompson and his colleagues have attempted to identify molecules that allow B cells to home to the bursa of Fabricius and be retained there. In collaboration with Dr. John Lowe (HHMI, University of Michigan), they have found that each of the stages of avian B cell development defined above is associated with a profound alteration in the glycosylation of the proteins and lipids on the surface of the cell. These changes in glycosylation are required for cells to 1) migrate to the bursa, 2) be retained within the bursal follicle,

and 3) migrate to the peripheral lymphoid organs following completion of the bursal-dependent phase of differentiation. How this differential glycosylation is regulated is now under active investigation.

Unlike mammals, the chicken immunoglobulin (Ig) loci contain only single variable and joining segments capable of undergoing rearrangement. To create an immunological repertoire, chickens must diversify the coding sequence of the Ig light-chain (Ig_L) and heavy-chain (Ig_H) V gene segments during development. This diversification occurs during the bursal-dependent phase of B cell development. Ig gene diversification is limited to the rearranged V gene segment and occurs by intrachromosomal gene conversion using V-region pseudogenes as sequence donors.

The laboratory has been able to demonstrate some novel features of this somatic gene conversion process. First, gene conversion appears to be dependent upon transcription of recipient sequences; second, cells that undergo gene conversion are proficient in homologous recombination; and third, gene conversion is restricted to the Ig loci.

To test whether Ig gene conversion is related to Ig rearrangement, the laboratory examined cells undergoing Ig_L gene conversion for the expression of genes required for Ig recombination and found that one gene, recombination-activating gene 2 (*RAG-2*), is selectively expressed during the bursal stage of B cell development. However, when the *RAG-2* gene was eliminated from a bursal cell line that constitutively undergoes Ig gene conversion in culture, the cell line retained the ability to undergo Ig gene conversion. Currently the laboratory is raising antisera to the RAG-2 protein in order to examine its role in the bursal-dependent phase of B cell development. (Studies of the regulation of Ig gene conversion are funded in part by a grant from the National Institutes of Health.)

Dr. Thompson is also Associate Professor of Medicine and of Microbiology and Immunology at the University of Michigan Medical School and a member of the Cell and Molecular Biology graduate program at the University of Michigan.

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MAMMALIAN MEMORY AND T LYMPHOCYTE FUNCTIONS

SUSUMU TONEGAWA, PH.D., *Investigator*

Dr. Tonegawa's laboratory has had a long-standing interest in the development and functions of T lymphocytes. While these immunological studies will actively continue, a new research interest, the mechanism of mammalian learning and memory processes, has taken clear shape in the laboratory during the past year.

Mammalian Memory

As a first step in a program to use genetically altered mice to study learning and memory mechanisms, Dr. Tonegawa and his co-workers, using the embryonic stem (ES) cell gene-targeting technique, produced mutant mice that do not express the α-calcium/calmodulin-dependent kinase II (α-CaMKII), an enzyme highly enriched in postsynaptic densities of hippocampus and neocortex. The mice exhibit mostly normal behaviors, no obvious neuroanatomical defects, and normal postsynaptic mechanisms, including N-methyl-D-aspartate (NMDA) receptor function. However, they were shown to be deficient in long-term potentiation (LTP) in the hip-

pocampus and to have specific learning impairments. These disabilities indicate that α-CaMKII has a prominent role in spatial learning but is not essential for some types of nonspatial learning. The data thus considerably strengthen the contention that the synaptic changes exhibited in LTP are the basis for spatial memory.

Dr. Tonegawa's laboratory is carrying out further characterization of the α-CaMKII mutant mice with respect to a possible defect in the modulation of the amygdala-based acoustic startle response (a collaboration with Dr. Michael Davis at Yale University) and to the kindling behavior (a collaboration with Dr. James McNamara at Duke University). Dr. Tonegawa's group is also producing several other mice, each with a mutation in a gene of neurobiological significance.

DNA Rearrangement

To test the hypothesis that somatic DNA rearrangement akin to immunoglobulin (Ig) or T cell receptor (TCR) V(D)J (V, variable; D, diversity; J, joining) recombination plays a pivotal role in the development or functions of the central nervous system (CNS), Dr. Tonegawa's laboratory produced transgenic (Tg) mice with the bacterial β-galactosidase gene (β-lacZ) whose expression would be dependent on a V(D)J or V(D)J-like inversional recombination occurring in its flanking regions. Despite the previous report of others to the contrary, Dr. Tonegawa's group obtained no evidence of somatic recombination in the CNS of the Tg mice. They concluded that the previously presented evidence is disputable and that somatic rearrangement in the CNS has yet to be demonstrated.

$\alpha\beta$ T Cells

Using the ES cell gene-targeting technique, Dr. Tonegawa's laboratory produced mice with mutations in the TCR α, β, or δ gene as well as in the RAG-1 gene, whose product is required for somatic rearrangement of Ig and TCR genes. Each mutation blocks T cell differentiation at a distinct stage. In RAG-1 mutants, the thymocyte differentiation is blocked at a CD4-CD8 double-negative (DN) state. Rearrangement and/or expression of the TCR β gene is required and sufficient for the transition of the DN to the double-positive (DP) state and for the expansion of the DP cells to the wild-type level. TCR α gene rearrangement and/or expression is irrelevant for these events but required for the transition of the DP to CD4 or CD8 single-positive state. Neither TCR α nor TCR β is required for the generation of $\gamma\delta$ T cells. Reciprocally, the TCR δ mutation does not block $\alpha\beta$ T cell differentiation.

While it is clear that TCR β gene rearrangement occurs prior to TCR α gene rearrangement in thymic ontogeny, neither is required for the other to occur. In the thymus and the lymph nodes of TCR α mutant mice, a small fraction of T cells express β only (i.e., α-less) TCRs associated with CD3 molecules. In the lymph nodes, these T cells are exclusively CD4-positive, suggesting that they interact with MHC class II molecules during differentiation. The functions of these novel T cells are unknown.

Dr. Tonegawa's laboratory also produced mice with mutations in the peptide-transport gene TAP-1. In these mice, cell surface expression of MHC class I molecules and the generation of CD8-positive T cells are severely impaired. Expression of an MHC class I molecule can be restored specifically in vivo or in vitro upon injection of or treatment with a peptide known to bind with that molecule. Thus these mutant mice provide an excellent system for studying both the role and the nature of self peptides involved during intrathymic positive selection of T cells. (This project has been supported in part by grants from the National Institutes of Health.)

$\gamma\delta$ T Cells

T cells with $\gamma\delta$ receptors are composed of subsets that utilize one or a few distinct V gene segments each to encode its TCR, which appears at a particular time in ontogeny. By making use of the fact that TCR γ and δ gene rearrangement can occur in the TCR δ mutant mice without cell surface expression of the receptor (and therefore without TCR-mediated cellular selection), Dr. Tonegawa's laboratory demonstrated that developmentally programmed TCR gene rearrangement plays a pivotal role in the temporarily differential generation of the $\gamma\delta$ T cell subsets.

Using the same mice, the laboratory also demonstrated that the remarkable sequence homogeneity in the V(D)J junctions of the TCR genes expressed in the skin- and uterus-associated $\gamma\delta$ T cell subsets is accomplished primarily by evolutionarily acquired sequence features at the junction of these genes and only auxiliarily by cellular selection.

On the other hand, the laboratory demonstrated the importance of cellular selection in the maturation of $\gamma\delta$ T cells in a Tg mouse system. In the thymus or the spleen of mice produced by crossing $\gamma\delta$ TCR–Tg mice with others genetically deficient in β_2-microglobulin (β_2m), no mature Tg $\gamma\delta$ T cells are generated. Hence interaction between the Tg $\gamma\delta$ TCR and a β_2m-associated molecule is required for the generation of mature Tg $\gamma\delta$ T cells.

Dr. Tonegawa and his colleagues used the TCR α and TCR β mutant mice to identify the function of $\gamma\delta$

T cells. In collaboration with Dr. Stefan Kaufmann's laboratory (University of Ulm), they showed that the TCR β mutant mice are as resistant to *Listeria monocytogenes* infection as the wild-type mice. *In vivo* treatment of the TCR β mutant mice with anti–γδ TCR antibody exacerbated *L. monocytogenes* by nearly 100-fold, suggesting a role for γδ T cells in resistance to this infection. A similar finding was made for vaccinated TCR β mutant mice against secondary *Listeria* infection.

In collaboration with Dr. Ruth Nussenzweig's laboratory (New York University), Dr. Tonegawa's laboratory also showed that mice vaccinated with mouse malaria-causing *Plasmodium yoelii* are partially resistant to secondary infection with the same parasite. A γδ T cell clone isolated from the spleen of the infected mice could confer a partial resistance to *P. yoelii*. (This project has been supported in part by grants from the National Institutes of Health.)

Dr. Tonegawa is also Professor of Biology at the Massachusetts Institute of Technology and at the Center for Cancer Research.

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CELL SURFACE MOLECULES AND MOLECULAR EVENTS INVOLVED IN HUMAN T CELL ACTIVATION

ARTHUR WEISS, M.D., PH.D., *Associate Investigator*

A T lymphocyte response to a specific antigen is initiated during a complex intercellular interaction with an antigen-presenting cell (APC), and central to this response is the T cell antigen receptor (TCR). The TCR has two functions: to recognize the antigenic peptide bound to a molecule of the major histocompatibility complex, and to convert this recognition event into a transmembrane signal initiating cellular differentiation and proliferation. Although the specificity of the response suggests that its initiation pivots on the interaction of the TCR with antigen, other molecules expressed on the plasma membrane are now seen to play important roles in regulating the activation of the T cell. In addition to the TCR, Dr. Weiss's laboratory has focused on the function of CD45 and CD28.

TCR Structure and Function

The TCR is an extraordinarily complex structure composed of at least eight chains, the products of six genes. The Ti subunit, a heterodimer of α and β chains derived from immunoglobulin (Ig)-like genes, is responsible for antigen recognition. Noncovalently associated with Ti in an obligatory manner are the invariant CD3 and ζ subunits. The CD3 subunit comprises three homologous chains, γ, δ, and two copies of ε. The ζ subunit is a homodimer or heterodimer of ζ and an alternatively spliced form of ζ, the η chain, or the γ chain of the IgE Fc receptor. The CD3 and ζ subunits couple the Ti subunit to intracellular signal transduction components.

The complex structure of the TCR has impeded a simple structure-function analysis. However, stud-

ies with chimeric molecules demonstrated that sequences contained within the transmembrane domains of the TCR chains are responsible for the association of the TCR subunits. Dr. Weiss and his colleagues took advantage of this information to develop a strategy to study the function of domains of individual TCR chains. A chimeric molecule in which the cytoplasmic domain of ζ was fused to the extracellular and transmembrane domains of CD8 was constructed and could be expressed in the absence of other TCR chains. This permitted the analysis of the function of the cytoplasmic domain of ζ independently of other TCR chains. Remarkably, stimulation of CD8/ζ induced all of the early and late events associated with stimulation of the intact oligomeric TCR.

More recent studies have identified a 22-residue sequence motif, based on conservatively placed tyrosine and leucine residues, that is sufficient to couple chimeric receptors to the TCR-regulated signal transduction events. This motif is triplicated within ζ and is contained as a single copy in all three CD3 chains as well as in the non-ligand-binding chains of the B cell antigen receptor and the mast cell IgE Fc receptor. Thus it appears that these oligomeric receptors expressed on distinct cell types of the hematopoietic lineage utilize a similar structural motif to couple to intracellular signal transduction components.

Stimulation of the TCR activates a protein-tyrosine kinase (PTK) not intrinsic to the structure of the receptor. Activation of this PTK induces the tyrosine phosphorylation of several proteins, including the TCR ζ chain, phospholipase C (PLC)-γ1, mitogen-activated protein kinase-2, and the proto-oncogene *Vav*. Dr. Weiss and his colleagues used signal transduction mutant cell lines to show that phosphorylation of PLC-γ1 is associated with its activation. (This work was supported by a grant from the National Institutes of Health.)

Since ζ can link the TCR to intracellular signal transduction components, experiments during the past year were directed toward identifying molecules that interact with the cytoplasmic domain of ζ. A 70-kDa tyrosine phosphoprotein rapidly associates with ζ following TCR stimulation. This protein, termed ZAP-70 (for ζ-associated protein), associates with ζ via sequences contained within the ζ activation motif, and this correlates with increased PTK activity in ζ immunoprecipitates from stimulated cells. Preliminary sequence and expression analyses reveal that ZAP-70 is a PTK. It is likely to be involved in TCR-mediated signal transduction.

Recent studies of J.CaM1, a previously isolated mutant cell line with a defect in TCR-mediated signal transduction, implicate another PTK in the pathway. (This work was supported by a grant from the National Institutes of Health.) Stimulation of the TCR on J.CaM1 fails to induce PTK or PLC activities. This cell has been found to be deficient in Lck PTK function as a result of an abnormally spliced Lck transcript. TCR signaling function was restored by transfection with Lck cDNA. Thus these studies implicate two distinct PTKs, ZAP-70 and Lck, in TCR signal transduction. It will be interesting to determine if these PTKs interact during the transduction process.

CD45 Tyrosine Phosphatase Regulates TCR Signal Transduction

CD45 is a transmembrane tyrosine phosphatase expressed on most hematopoietic cells. Various isoforms of CD45, the products of alternative splicing of the same gene, are expressed in a tissue and activation manner. Mutant cell lines deficient in CD45 expression have defects in TCR-mediated induction of PTK and PLC activities. The signaling functions of these mutants have now been reconstituted with either the 220- or 180-kDa isoforms of CD45. Work is in progress to assess the role of the extracellular domain in regulating CD45 function.

Of considerable interest is the identification of the relevant intracellular targets of CD45. Potential targets of CD45 include members of the Src family of PTKs. These PTKs each have a negative regulatory tyrosine phosphorylation site near their carboxyl termini. Thus the signaling defect in CD45-deficient cells might involve a Src PTK family member critical in TCR-mediated signal transduction that is inactive because of hyperphosphorylation at its negative regulatory site. Indeed, preliminary assays of Lck isolated from CD45-deficient cells reveal it to be hyperphosphorylated at this site. Thus a PTK shown to be required for TCR-mediated signal transduction in the J.CaM1 mutant is also negatively regulated in CD45-deficient cells. Future experiments are aimed at determining whether the status of Lck phosphorylation is the sole explanation for the signal transduction deficiency in CD45-deficient cells.

CD28 Regulation of Lymphokine Gene Expression

Stimulation of the TCR alone does not suffice to initiate lymphokine gene expression or T cell proliferation. Stimulation of other receptors, such as CD28, provides a co-stimulatory function. CD28 binds to B7, a molecule expressed on potent APCs

such as activated B cells and macrophages. CD28 induces the activity of a nuclear complex, CD28RC, that binds to a site CD28 in the interleukin-2 (IL-2) gene, CD28RE, and regulates promoter activity. During the past year the CD28-B7 interaction during physiologic stimulation of T cells by superantigen was shown to play a critical role in influencing IL-2 promoter activity through interactions that depend on the CD28RE. (This work was supported by a grant from the National Institutes of Health.) Thus different signal transduction pathways regulated by distinct T cell surface molecules, the TCR and CD28, coordinately regulate the activation of the IL-2 gene.

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LYMPHOCYTE-SPECIFIC GENES IN NORMAL AND NEOPLASTIC LYMPHOCYTE DIFFERENTIATION

Irving L. Weissman, M.D., *Investigator*

Lymphocyte Homing Receptors

Lymphocytes are mobile cells that move from organ to organ via the blood and lymphatic vascular systems. Most mature blood lymphocytes are en route to lymphoid organs, which they enter within minutes after their appearance in the blood.

Twenty-five years ago, Dr. Weissman and his colleagues reported that new thymic emigrants enter lymphoid organs via organ-specific high-walled endothelial vessels (HEVs); and later, that both B and T lymphocytes enter lymphoid organs via common HEVs, but that lymphocytes use different homing receptors to bind to Peyer's patch versus lymph node HEVs. Using monoclonal antibodies, they identified and characterized the protein that makes up the major lymph node homing receptor (LNHR) molecule and the Peyer's patch homing receptor (PPHR) heterodimeric protein molecules. They then cloned the cDNA encoding mouse and human LNHRs and found that it was the first member of a new adhesion family called the selectins.

During the past year, Dr. Weissman's group cloned the genes encoding the two chains of the PPHR, $\alpha 4$ and $\beta 7$. The $\alpha 4$ chain is found not only in the PPHR, but is part of the VLA4 molecule that the group showed to be involved in the binding of hematopoietic precursors to thymic and bone marrow stromal cells and to inflamed endothelium. The cDNA encoding the $\alpha 4$ integrin contains sequences shared by other Ca^{2+}-binding proteins that likely are involved in the noncovalent, Ca^{2+}-dependent association of $\alpha 4$ with $\beta 7$ or $\beta 1$ chains. The cDNA encoding the $\beta 7$ gene also has two putative Ca^{2+}-binding domains, one an amino-terminal E-F hand sequence and the other a tetrad of EGF-similar sequences.

Several regions of the murine and human $\alpha 4$ cDNAs are conserved, most strikingly the transmembrane and short cytoplasmic domains. (All three homing receptor genes [L-selectin, $\alpha 4$, $\beta 7$] contain transmembrane and cytoplasmic sequences conserved between mouse and human.) Transfection of the L-selectin leads to the homing of transfected lymphomas to lymph nodes but not Peyer's patches. Transfection of $\alpha 4$ or $\beta 7$ alone does not result in Peyer's patch homing, while transfection of both $\alpha 4$ and $\beta 7$ does.

Role of Killer T Cells and Natural Killer Cell–Specific Genes in Normal and Pathological Immunocellular Reactions

Several years ago Dr. Weissman's laboratory cloned a cDNA encoding a gene selectively expressed in the vesicles of killer T lymphocytes and natural killer cells. The protein product, called granzyme A (GrA), is a homodimeric serine protease with trypsin-like specificity. GrA, other proteases, and perforin are released at the interface of killer cell–target cell junctions, causing target cell lysis. In the past year the laboratory demonstrated that a high proportion of cells in synovial fluid of patients with active rheumatoid arthritis are perforin and GrA positive. This is the first demonstration that cells with putative cytolytic capacity might be involved in the cytodestructive lesions of rheumatoid arthritis. In an examination of a number of skin lesions involving lymphocytic infiltration, it was shown that most do not contain cells expressing perforin or GrA, even in lesions wherein CD8+ cells predominate.

To test directly the association of killing and GrA positivity, the laboratory carried out a number of experiments wherein rat cardiac allografts present in mice and rats pretreated with anti-CD4 or cyclosporin-A (CsA) were analyzed. The anti-CD4– and the CsA-treated animals are tolerant but contain infiltrates of cells devoid of GrA and/or perforin message. These studies provide indirect evidence that perforin ± GrA+ cells are involved in cytolytic processes.

Killer cells lyse target cells by two apparently independent mechanisms: formation of membrane pores leading to leakage of cytoplasmic contents, and induced apoptosis (suicide) resulting in degradation of chromosomal DNA into nucleosomal-size fragments. Dr. Pierre Henkart has proposed that GrA is responsible for induction of apoptosis. In experiments in collaboration with Dr. Lishan Su in Dr. Weissman's group, transfection of GrA into mast cells already expressing perforin endows these cells with apoptosis-inducing capacity.

Apoptosis may also occur in lymphocytes treated with radiation or hydrocortisone or in cell lines dependent on cytokines and then deprived of them. In these cases, transfection of the bcl-2 gene prevents apoptosis. Despite the similarity of hydrocortisone-induced or cytokine-removal–induced apoptosis to cytolytic cell–induced apoptosis, Dr. Weissman's laboratory found that bcl-2–transfected cells are not resistant to killer cell–induced apoptosis, even if they are resistant to apoptosis induced by cytokine removal.

The laboratory examined the genomic sequence for the GrA gene and compared it with its mRNA sequences. Two cDNA sequences representing the products, respectively, of the 5'-most exon (EI) alone or that exon and its adjacent 3' exon (EI, EII) were represented in two RNA species. Expression of EI should give a hydrophobic leader to place the protein in the endoplasmic reticulum for transport to the vesicle; expression of EI, EII should result in a protein lacking the hydrophobic sequence, the result of an EII termination codon followed by an ATG. The EII sequence lacks the hydrophobic characteristics of a signal sequence, and it is very likely that the protein would be expressed in the cytoplasm. This EI, EII sequence can be found in a subset of cytolytic clones and in the thymus.

Because secreted GrA triggers apoptosis in target cells, it is intriguing to consider the possibility that the appearance of the EI, EII form of GrA in the cytoplasm of killer cells and/or a subset of thymocytes might induce apoptosis in these cells. In this case, such expression could be involved in determining the life span of these cells. Programmed cell death and programmed organismal death, by known genes and/or gene products have therefore become subjects of importance to this laboratory. Their study could be useful in understanding the regulation of life span as well as the mechanism by which the absence of positive selection and/or the presence of negative selection cause(s) death in a subset of developing thymocytes.

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NORMAL AND ABNORMAL LYMPHOCYTE GROWTH REGULATION

Owen N. Witte, M.D., *Investigator*

The proper functioning of the immune system depends on the careful regulation of lymphocyte production in the bone marrow. Pluripotential stem cells divide and develop into committed progenitors for the B and T lymphoid lineages. Abnormal regulation of this process can lead to hyperproliferative states such as leukemia or hypoproliferative states such as immunodeficiencies. A major goal of Dr. Witte's laboratory has been to devise techniques for the isolation and characterization of such stem and progenitor cells.

B Lymphocyte Development

Previous work established culture conditions for the continuous cultivation of immature murine B lymphocytes and their progenitors. Recent improvements in these culture conditions have allowed a significant enrichment of progenitor cells for the B cell lineage. These cells express known B cell surface markers and retain immunoglobulin genes in the germ configuration. The progenitor cells are responsive to a new growth factor produced by stromal cells in the culture system. Previously described growth factors known to stimulate pre–B lymphocytes, including the KIT ligand and interleukin-7, are incapable of stimulating the B lineage progenitor cells.

Using such progenitor cells as a source of mRNA, Dr. Witte and his colleagues have isolated several B lineage–specific genes that are expressed at this early point in development. One in particular, a new member of the cytoplasmic tyrosine kinase family, has been evaluated in detail. This protein, B lineage progenitor kinase (BPK), helps to define a new subclass of tyrosine-specific protein kinases. Although generally related to the *src* family of kinases by the presence of the tyrosine kinase catalytic domain and the SH2 and SH3 regulatory domains, the BPK kinase has an unusually long amino-terminal segment and lacks the carboxyl-terminal regulatory domain found in the *src* family kinases. The precise role of this kinase in B cell development is under investigation.

Further analysis of the relationship of B cell progenitors to pluri- and multipotential stem cells requires the development of stage-specific markers. A new project proposes to develop a large panel of hematopoietic-specific genes that can be assigned to different lineages and cell types. In collaboration with Dr. Leroy Hood, phage libraries were derived from human bone marrow RNA. Clones enriched to represent rare or moderately expressed mRNAs were isolated. Each cloned sequence defines a new gene that was monitored for pattern of expression by hybridization against a large panel of leukemic cell RNAs. With this approach one should be able to assemble a panel of markers specific for different cell lineages and stages of maturation. Eventually, selected probes will be evaluated in greater depth on normal marrow specimens.

Use of Oncogenes to Stimulate Primitive Hematopoietic Stem Cells

Previous work from this laboratory had defined the *BCR/ABL* oncogene expressed in human chronic myelogenous leukemia as a valuable tool for the transformation of primitive lymphoid cells and myeloid cell types. Recent work has shown that this gene can also be introduced into murine pluri- or multipotential stem cells to create a syndrome with many of the hallmarks of chronic myelogenous leukemia. Further analysis has demonstrated that direct stimulation of the stem cell can be achieved *in vitro*, as monitored in agar colony assays. The growth stimulation imparted by this gene is rather subtle, and there is no block to differentiation of the cells. Thus this relatively weak oncogene can be used to expand populations of cells that retain full developmental potential.

Analysis of the mechanisms of activation of the *BCR/ABL* gene has demonstrated that unique features of the BCR segment are required to interact with the ABL tyrosine kinase segment to achieve transformation activation. The BCR segment contains a serine-rich segment that can bind to the tyrosine kinase SH2 regulatory domain. This putative cis interaction between the segments of the chimeric oncogene appears to be critical for the activation of the ABL tyrosine kinase. Site-directed mutagenesis has shown that the ABL tyrosine kinase and a major site of tyrosine phosphorylation within ABL are also essential for full transformation. (This work was supported by a grant from the National Cancer Institute.)

To provide better test models for the analysis of human leukemias, tissue explants of peripheral blood and bone marrow have been cultured in the *in vivo* environment of the SCID (severe combined immune deficiency) mouse strain. The results are dramatic. All specimens of acute myelogenous leukemia and all blast crisis specimens of chronic myelogenous leukemia grew well in the SCID mouse.

This is a significant improvement over cell culture strategies in which only a rare leukemia can be coaxed into continuous growth. The SCID mouse should be an important test model for evaluation of new therapeutic modalities in chronic and other myelogenous leukemias.

The unusual mechanism by which BCR appears to activate ABL stimulated further work to define the range of genetic alterations that can activate this member of the tyrosine kinase family. Alteration of the regulatory domain SH3 by deletion or insertion mutation was previously shown to activate the transforming potential of the ABL tyrosine kinase. Recent work in this laboratory has now demonstrated that exchange of SH2 domains with other members of the tyrosine kinase family or from non-kinase SH2-containing proteins is also sufficient to activate the ABL tyrosine kinase. This suggests that subtle changes in structure within these regulatory domains is sufficient for activation and should be considered in other abnormal growth states associated with the *ABL* gene.

Dr. Witte is also Professor of Microbiology and Molecular Genetics, holds the David Saxon Presidential Chair in Developmental Immunology, and is a member of the Molecular Biology Institute at the University of California, Los Angeles.

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NEUROSCIENCE

The Institute's Program in Neuroscience was established in 1984 in recognition of the challenging biomedical questions and research opportunities posed by the human brain and its function. Much of the research in this program is focused on the ways in which nerve cells conduct signals and communicate with each other and with the effector tissues of the body (such as muscle and gland cells), on the cellular mechanisms involved in the development of the nervous system, and most recently, on cognitive neuroscience and the computational capacity of complex neural networks.

Investigators in the neuroscience program area are working at Johns Hopkins University, Massachusetts General Hospital in Boston, the Massachusetts Institute of Technology, the University of Texas Southwestern Medical Center at Dallas, Yale University, Columbia University, New York University, Stanford University, the California Institute of Technology, the Salk Institute for Biological Studies, the University of Washington in Seattle, the State University of New York at Stony Brook, Rockefeller University, Brandeis University in Waltham, and the University of California at Berkeley, Los Angeles, San Diego, and San Francisco.

Neurons communicate with each other and with other cells through small molecules, the neurotransmitters. This communication occurs at specialized contact zones, the synapses. At the synapse, one cell (the presynaptic neuron) sends out a chemical signal that is recognized by the second, postsynaptic cell by means of receptors. This process of neurotransmission represents one of the bases for information processing in the brain. Neurotransmission is subject to extensive regulation that includes some forms of learning and memory, and disturbances of neurotransmission are thought to be involved in several diseases of the nervous system.

The laboratory of Investigator Thomas C. Südhof, M.D. (University of Texas Southwestern Medical Center at Dallas) is studying how cells form presynaptic nerve terminals and how the nerve terminals send out chemical signals at synapses. The work focuses on the cellular organelles in the presynaptic cells that store the chemical signals used for synaptic communication. Experiments are designed to determine how the chemical signals are released, how this release is regulated, and how this release is targeted exclusively to the site of the cell where the postsynaptic nerve cell awaits the signal. The work has led to the characterization of several novel proteins in the presynaptic cell that are likely to be involved in neurotransmission. In addition, a new family of cell surface receptors that may function in determining specific connections between cells has also been discovered. The results are relevant for understanding basic cellular processes (such as how cells secrete chemicals) as well as for insight into complex physiological processes (such as learning and memory).

The research in the laboratory of Associate Investigator Richard L. Huganir, Ph.D. (Johns Hopkins University) is directed toward the understanding of the molecular mechanisms that underlie the modulation of synaptic function. Specifically, this group is investigating the role of protein phosphorylation in the regulation of neurotransmitter receptor function. The laboratory has used the nicotinic acetylcholine receptor, the prototypic neurotransmitter receptor, as a model system and has found that the nicotinic receptor is phosphorylated at seven different sites by three different protein kinases. Phosphorylation of the nicotinic acetylcholine receptor enhances its desensitization to its neurotransmitter, acetylcholine. The phosphorylation (and thus the desensitization) of the nicotinic receptor by these three protein kinase systems is under the control of at least three neurotransmitters. In addition, recent results have suggested that tyrosine phosphorylation of the receptor may regulate receptor aggregation at the synapse during its formation. Dr. Huganir's laboratory has also been investigating the role of protein phosphorylation in the regulation of the major excitatory and inhibitory neurotransmitter receptors in the brain, the glutamate receptors and $GABA_A$ receptors. Results suggest that protein phosphorylation of neurotransmitter receptors is one of the major mechanisms in the modulation of their function and may play an important role in synaptic plasticity, such as long-term potentiation and long-term depression.

Investigator Pietro De Camilli, M.D. (Yale University) and his colleagues are interested in elucidating the traffic of synaptic vesicles (SVs) in neurons. Work carried out during the past year has provided further support for the hypothesis that SVs represent the neuronal adaptation of organelles found also in endocrine cells: synaptic-like microvesicles. It was shown that synaptic-like microvesicles of β cells store the neurotransmitter GABA. A GDP-releasing protein that may play a role in exocytosis from mammalian cells, including neurons, was identified and

characterized. SV-associated proteins that become autoantigens in the human stiff-man syndrome were characterized.

During the process of neuron communication, the sender cell, upon excitation, releases neurotransmitters by calcium-dependent exocytosis of synaptic vesicles, and the mechanisms of neurotransmitter storage and release are under study by Associate Investigator Reinhard Jahn, Ph.D. (Yale University) and his colleagues. Synaptic vesicles are retrieved by endocytosis and are re-formed within the nerve terminal after passing through intermediate stages that probably include clathrin-coated vesicles and endosomes. Synaptic vesicles contain a group of specific membrane protein families with unique structural properties. The vesicle protein synaptotagmin was found to bind calcium ions at physiologically relevant concentrations, accompanied by an interaction with phospholipid membranes. Therefore the protein is a candidate for being the exocytotic calcium ion receptor. The role of small GTP-binding proteins of the ras superfamily in vesicular membrane traffic was examined. Several of the proteins appear to function in exocytosis and membrane recycling of synaptic vesicles. Their mechanism of action remains to be clarified, but they may control individual steps of the vesicle cycle.

Associate Investigator Richard H. Scheller, Ph.D. (Stanford University) and his colleagues examine the molecular mechanisms of brain development and function. They are particularly interested in the formation and function of the synapse, the specialized region of cells responsible for converting the electrical impulse of an action potential into a chemical signal that travels between cells. Many diseases are the result of synaptic dysfunction, and many drugs act to modulate various aspects of synapse function. Agrin, a protein localized at the nerve muscle synapse, acts to concentrate neurotransmitter receptors in the proper place during development. Dr. Scheller's group has characterized the agrin protein and gene. They have now turned their attention to the mechanism of agrin action during mammalian development. Many of the molecules associated with synaptic vesicles are thought to regulate the release of neurotransmitters. To understand these molecules better, the group has isolated cDNAs encoding the proteins. They are studying the roles of synaptic vesicle–associated proteins in the central nervous system.

Senior Investigator Eric R. Kandel, M.D. (Columbia University) and his colleagues study elementary forms of learning and memory. During the past year they focused on the molecular switch that leads to the activation of long-term memory storage in *Aply-*

sia. In addition, they have begun to explore some molecular mechanisms contributing to long-term potentiation in the hippocampus, a model of learning in the mammalian brain.

Learning is thought to involve the strengthening of specific synapses, the points of contact at which one nerve cell transfers information to another, so that this information transfer between nerve cells becomes more efficient. A phenomenon called long-term potentiation (LTP), intensively studied by neurobiologists, represents a long-lasting strengthening of synapses that occurs when those synapses are used repeatedly. LTP is widely believed to represent the cellular change underlying learning, but the evidence for this remains equivocal. Mutant mice have been produced that lack a single enzyme that normally is concentrated at synapses. These mice are found to be deficient in LTP and also in a particular type of learning associated with the brain structures in which LTP is usually most prominent. These experiments of Investigator Charles F. Stevens, M.D., Ph.D. (Salk Institute) and his colleagues, including the laboratories of Dr. Susumu Tonegawa and of Dr. Jeanne Wehner, support the hypotheses that LTP is a cellular mechanism of memory.

Studies in the laboratory of Investigator Thomas M. Jessell, Ph.D. (Columbia University) over the past year have focused on the cellular interactions that control the early organization of the vertebrate nervous system. The identity of cells and their position within the neural tube appear to be controlled by inductive signals that derive from two ventral midline cell groups, the notochord and floor plate. Removal of these two cell groups leads to the loss of ventral cell types, and placing them adjacent to the dorsal neural tube leads to the ventralization of dorsal regions. To understand the mechanisms by which cell identity and positions are determined by midline-derived signals, a search has been performed for genes that control the identity of floor plate cells and motoneurons. Two transcription factors have been identified that define early stages of floor plate and motoneuron differentiation, and misexpression of one of these genes leads to changes in neural cell fate. In addition, a novel growth factor has been identified that is expressed in the neural tube and is a member of the transforming growth factor-β family. This factor is restricted to dorsal regions of the neural tube and may be involved in the control of cell differentiation along the dorsoventral axis of the neural tube.

Investigator Edward B. Ziff, Ph.D. (New York University) and his colleagues have studied the regulation of gene activity during neuronal differentiation

induced in PC12 cells by nerve growth factor (NGF). They have shown that NGF induces the immediate-early c-*fos* gene, which encodes a transcription factor, c-Fos. Other members of the Fos family are also induced. The c-Fos stimulates a second gene encoding tyrosine hydroxylase (TH), an enzyme that catalyzes the rate-limiting step in dopamine neurotransmitter synthesis. The group has shown that a binding site for c-Fos (in a complex with c-Jun) in the TH promoter is critical for TH gene activation by NGF. The c-Fos may be an intermediary for TH gene regulation by signals from the plasma membrane. The E1a protein of adenovirus-5, which sequesters the tumor-suppressor protein Rb and related factors, was found to disrupt the neuronal phenotype of PC12. Dr. Ziff's laboratory is studying the role of tumor-suppressor proteins and nuclear proto-oncoproteins such as c-Fos and c-Myc in regulating neuronal differentiation.

Investigator Louis F. Reichardt, Ph.D. (University of California, San Francisco) and his colleagues are investigating molecules in the extracellular environment of neurons that regulate their survival and direct their development *in vivo*. One project seeks an understanding of the role of trophic factors in regulating neuronal survival and development. These are proteins that regulate the differentiation of several neuronal populations, including neurons that are important in cognition. A second project concerns molecules on the surfaces of cells and in the extracellular matrix that serve as substrates for guiding the growth of axons. These molecules play critical roles in establishing correct wiring of the nervous system. A third project is directed toward identifying molecules that direct neurons to form synapses with other cells. Synapses are the sites of information transfer between neurons, so these molecules are also crucial in establishing correct wiring of the nervous system. All of these studies are potentially useful in understanding and developing treatments for many diseases and disorders that disrupt normal brain function.

The laboratory of Assistant Investigator David J. Anderson, Ph.D. (California Institute of Technology) studies the development of the neural crest, a structure that generates the neurons and glia of the vertebrate peripheral nervous system. Candidate master regulatory genes, that may control early determinative steps in neural crest development, have been isolated by their homology to neurogenic determination genes in the fruit fly *Drosophila*. The regulation and function of these genes are being explored in detail. The group has also isolated embryonic precursors of chromaffin cells, the endocrine cells of the adrenal gland, and found that their development is controlled both by steroid hormones and by an internal clock.

The electrical activity of nerve cells arises as a result of the complex but carefully orchestrated opening and closing of thousands of the special protein molecules called ion channels. These are of five main types: sodium, potassium, calcium, chloride, and nonspecific cation channels, named according to the ions that can pass through the open channel. Their activity is regulated by their chemical and electrical environment. For example, a particular type of potassium channel that the laboratory of Investigator Paul R. Adams, Ph.D. (State University of New York at Stony Brook) studies is the M channel. It is opened by positive changes in membrane potential and by small increases in intracellular calcium. It is closed by neurotransmitters released by other nerve cells and by large increases in internal calcium. In combination with a plethora of other types of ion channels, it regulates the firing patterns of nerve cells.

The research of Associate Investigator Richard W. Aldrich, Ph.D. (Stanford University) and his colleagues is directed toward understanding the molecular mechanisms of electrical signaling in the nervous system. The molecular elements of electrical signaling are proteins that lie in the cell membrane and serve as ion channels. These proteins regulate the passage of electrical current into the cell in response to stimuli. In the past few years, work by the group has focused on the mechanisms whereby ion channels open and inactivate, or turn off, after excitation. They have found a number of specific regions in a particular channel protein that are involved in activation and inactivation, and have determined some of the biophysical mechanisms that underlie the operation of these proteins.

Voltage-sensitive potassium channels represent a diverse group of ion channels found in most cell types studied in the animal and the plant kingdom. In the nervous system, they control excitability and modulate the strength of synaptic inputs; some of the potassium channels have been implicated in the processes of learning and memory. The first potassium channel was cloned in the laboratory of Investigator Lily Y. Jan, Ph.D. (University of California, San Francisco) by exploiting *Drosophila* genetics. The initial molecular characterizations have provided some clues to questions concerning potassium channel diversity and have revealed some of the structural elements involved in different channel functions.

The laboratory of Investigator Christopher Miller, Ph.D. (Brandeis University) is interested in the basic mechanisms by which ion channel proteins

operate. Because these proteins are directly responsible for the ability of nerve cells to generate electrical signals, they lie at the molecular foundations of the nervous system. Current research by this group is aimed at the structure and function of potassium-specific channels, the purification of chloride-specific channels, and the interaction of certain channels with peptide neurotoxins.

The research of Investigator Roger Y. Tsien, Ph.D. (University of California, San Diego) and his colleagues focuses on intracellular signal transduction and new molecules to probe these biochemical mechanisms. Many hormones and neurotransmitters influence gene expression through the translocation of the catalytic subunit of cAMP-dependent protein kinase into the nucleus. This translocation was surprisingly found to be independent of kinase activity and mediated by passive diffusion. Preliminary evidence was found for a novel membrane-permeant messenger mediating calcium influx, whereas another messenger previously thought to have that role seems to depress intracellular calcium or uncouple it from downstream physiological functions. New molecules were devised for photochemically controlled release of intracellular calcium and the intercellular messenger nitric oxide.

Transmembrane signal transduction plays a key role in cellular physiology, growth, development, and differentiation. The most widespread transmembrane signaling system involves a superfamily of related G proteins and G protein–coupled membrane receptors. The laboratory of Assistant Investigator Thomas P. Sakmar, M.D. (Rockefeller University) employs the visual proteins transducin and rhodopsin as a model system for structure-function studies on the molecular mechanism of transmembrane signaling. The key approach has been to reconstitute heterologously expressed rhodopsin and transducin *in vitro* under defined conditions and to use biochemical and biophysical methods to probe site-directed mutants. For example, ultraviolet-visible spectroscopy of mutant pigments has helped to identify specific amino acids that determine the spectral properties of rhodopsin and the green and red human color pigments. The question of how a photochemical signal is transmitted from the core of rhodopsin to its surface where transducin becomes activated has also been addressed using a method that allows measurements of rhodopsin-transducin interactions by intrinsic fluorescence. Mixtures of rhodopsin and transducin can be assayed, and the effects of specific mutations can be evaluated. These studies may lead to a better understanding at a molecular level of how receptors activate their respective G proteins.

Visual sensory cells have characteristic responses that include photo-induced excitation followed by recovery and adaptation. Associate Investigator James B. Hurley, Ph.D. (University of Washington) is exploring the fundamental mechanisms of visual phototransduction. The roles of vertebrate photoreceptor proteins such as transducin and recoverin in recovery and adaptation were elucidated by a combination of genetic and biochemical investigations. A unique type of G protein subunit was identified in *Drosophila* photoreceptors, and it was shown to play a critical role in *Drosophila* phototransduction. The aim of these studies is to understand the molecular mechanisms that generate and regulate the primary visual response.

In the past year, Investigator King-Wai Yau, Ph.D. (Johns Hopkins University) and his colleagues continued to focus on the phototransduction process in the retina. A major finding is that the cGMP-gated cation channel that mediates this process appears to be a hetero-oligomer, rather than a homo-oligomer as had previously been thought. They have cloned an apparently new species of this channel subunit that by itself is unable to form a functional channel but when coexpressed with another subunit confers the flickering channel kinetics that are characteristics of the native channel.

Recent research has uncovered the existence of a specialized pathway in the cerebral cortex that appears to process information about visual motion. Signals about visual motion form the foundation of many aspects of higher-order visual analysis, and the laboratory of Investigator J. Anthony Movshon, Ph.D. (New York University) has been exploring several ways in which these signals support perceptual decisions and visuomotor behavior. By combining electrophysiological recording with perceptual experiments in awake trained monkeys, the group has shown that despite an available large pool of neurons in the cortical motion pathway, the information carried by small numbers is sufficient to support perceptual performance. This finding suggests that it may be neither necessary nor desirable for the brain to pool large numbers of distributed neuronal signals to arrive at perceptual decisions. Signals carried by these same cortical neurons are thought to provide information to the motor system for the generation of smooth pursuit eye movements, used by primates to stabilize the retinal image of attended moving visual targets. Analysis of the dynamics of these rapid and precise movements suggests that visual motion signals may carry information about target speed, direction, and acceleration.

The research in the laboratory of Investigator Terrence J. Sejnowski, Ph.D. (Salk Institute) on mod-

eling the visual system has focused on two properties of visual stimuli—disparity and motion. During the development of the visual cortex, cortical neurons are sensitive to correlations in the electrical activity coming from the two eyes. The laboratory studied a computer model for the development of disparity-selective cells in the visual cortex that depended on prenatal and postnatal phases. The model mimicked the observed relationship between disparity and ocular dominance. Models were also examined for the representation of egocentric distance in a population of disparity-tuned neurons that receive eye vergence signals. The neurons in area MT of monkeys respond selectively to the motion of visual stimuli, but the same neurons are also tuned for other properties such as spatial frequency and disparity. A new approach to the integration of motion signals in area MT was introduced that depends on estimating the reliability of the local motion information. These estimates can be made by a separate population of neurons whose properties are predicted by the model.

Associate Investigator David P. Corey, Ph.D. (Massachusetts General Hospital) and his colleagues have been studying the processes whereby the ion channels in the sensory receptor cells of the inner ear are activated in response to sound. In the past year this laboratory has found that the adaptation mechanism in vertebrate hair cells of the inner ear only works over a narrow range. The structure responsible for adaptation is apparently the density marking the tip-link attachment site, since it moves with adaptation but its movement is limited. In other work the inherited human disease, hyperkalemic periodic paralysis, was shown by several criteria to be caused by a defective sodium channel in muscle.

The laboratory of Investigator Richard Axel, M.D. (Columbia University) is interested in the sense of smell. Mammals possess an olfactory system with enormous discriminatory power. How are the diversity and specificity of olfactory perception accomplished? In initial experiments to define the logic underlying olfactory perception, Drs. Axel and Linda Buck identified and cloned a large family of genes that are likely to encode odorant receptors in vertebrates. Analysis of the structure of this large gene family and examination of the patterns of expression provide insight into the mechanism by which the olfactory system can recognize a diverse array of odorants and how the brain can discriminate among different odors.

The mammalian olfactory system can discriminate among more than 10,000 different odors at concentrations as low as a few parts per trillion in air. The

laboratory of Associate Investigator Randall R. Reed, Ph.D. (Johns Hopkins University) has identified several of the proteins thought to be responsible for signal transduction in the olfactory system. Olfaction and vision are thought to share analogous biochemical mechanisms for converting external stimuli into electrical signals. The pathway for olfactory signal transduction appears to utilize a G protein cascade coupled to adenylyl cyclase. This laboratory has identified specialized forms of G proteins, adenylyl cyclase, and ion channels that are believed to mediate olfactory signal transduction. A recently described family of G protein–coupled receptors may contribute to the diversity in ligand recognition associated with this sensory system. Recently this group has examined the subcellular localization of these putative receptors and the patterns of expression of individual members of this gene family in olfactory epithelium. The laboratory also uses molecular components of mammalian signal transduction pathways as probes to identify homologues in the genetically manipulable organism *Drosophila melanogaster*.

Research in the laboratory of Associate Investigator Steven A. Siegelbaum, Ph.D. (Columbia University) concerns the mechanisms whereby intracellular second messenger molecules regulate neuronal function by controlling the activity of membrane ion channels. During the past year this group has focused on studies of cyclic nucleotide–gated channels involved in olfactory signal transduction. They have characterized the properties of a cloned cyclic nucleotide–gated channel from catfish olfactory neurons and have compared the properties of the cloned channel expressed in *Xenopus* oocytes with the native channel in catfish olfactory neurons. In addition, they have shown that the cyclic nucleotide–gated channel becomes less active when the intracellular calcium concentration is elevated. This inhibition is likely to play an important role in sensory adaptation, a process in which a maintained exposure to an odorant leads to a decline in response.

The superfamily of seven-transmembrane domain receptors linked to G proteins provides a major biological mechanism for detecting intercellular and environmental signals. Because the receptors are often situated at control points for crucial cellular activities, understanding the molecular basis of how ligands interact with them is important for the development of therapeutically useful chemicals. To address this problem, Associate Investigator Michael R. Lerner, M.D., Ph.D. (Yale University) and his colleagues have developed a versatile G protein–coupled, receptor bioassay. It is based on melano-

phores, the cells that many animals use to change their color rapidly. The bioassay will be useful for examining how ligands stimulate or block seven-transmembrane domain receptors.

Analyses of steroid receptors are important for understanding molecular details of transcriptional control, as well as providing insight as to how an individual trans-acting factor contributes to cell identity and function. Recent work from the laboratory of Investigator Ronald M. Evans, Ph.D. (Salk Institute) points to the existence of a specific molecular mechanism in the form of a physiologic code built into DNA that provides a direct structural correlate to the distinct hormonal signaling pathways for retinoic acid, vitamin D, and thyroid hormones. Although molecular biologic techniques have led to the discovery of new receptors, these discoveries, in turn, have enabled the identification of novel ligands for these receptors, as exemplified by the recent characterization by this laboratory of the new mammalian hormone 9-*cis* retinoic acid. It is believed that this may be a major physiologic regulator in the embryo and the adult and has recently been shown to have promise for the treatment of human acute promyelocytic leukemia.

Understanding the molecular mechanisms by which the neuroendocrine system develops and by which neuropeptides and hormones control critical regulatory events is the central goal in the laboratory of Investigator Michael G. Rosenfeld, M.D. (University of California, San Diego). Synergistic interactions required to produce the precisely restricted patterns of cell-specific expression have been identified for several pituitary genes. The cloning of DNAs complementary to the mRNAs that encode pituitary-specific transcriptional activators has provided new insights into organogenesis and characterized novel classes of transcriptional activators. A large number of structurally related members of this gene family have been identified in the brain and exhibit precise temporal and spatial patterns of expression in the developing nervous system. Detailed structure-function analysis of several hormone receptors has provided specific insights into the molecular mechanisms of receptor-mediated activation of gene expression.

The nervous system contains a diverse group of cells. What tells a cell to become a neuron? How does each neuron acquire its own unique identity? In the past few years the research group of Investigator Yuh Nung Jan, Ph.D. (University of California, San Francisco) and other laboratories have identified more than 20 genes that appear to have important roles in neurogenesis or in specifying neuronal identity. During the past year, Dr. Jan's group has

studied several of these genes in detail: *asense, big brain, cut, daughterless, deadpan, neuralized, numb, prospero*, and *rhomboid*. All have now been cloned. These studies are revealing clues as to how these genes affect neural development at the molecular level, as well as unexpected links between different developmental processes.

Taking a primarily genetic approach to answer the question of how genes control animal development, members of the laboratory of Investigator H. Robert Horvitz, Ph.D. (Massachusetts Institute of Technology) have isolated developmental mutants of the nematode *Caenorhabditis elegans* and have characterized them using both genetic and molecular techniques. Because both the complete cellular anatomy (including the full wiring diagram of the nervous system) and the complete cell lineage of this nematode are known, mutant animals can be studied at the level of single cells, and even single synapses. In this way, genes involved in cell lineage, cell signaling, cell death, cell migration, and cell differentiation have been identified and analyzed.

The so-called "*Notch* group" of genes has been implicated in the general mechanism that is crucial for correct developmental choices in a wide variety of precursor cells in *Drosophila* and is under study by Investigator Spyridon Artavanis-Tsakonas, Ph.D. (Yale University) and his colleagues. The accumulated genetic and molecular studies suggest that these genes encode elements of a cell communication mechanism that includes cell surface, cytoplasmic, and nuclear components. The central player of the *Notch* group is the *Notch* (*N*) locus, which encodes a transmembrane protein containing EGF-like (epidermal growth factor-like) repeats in its extracellular domain. Evidence suggests that this protein may act as a multifunctional receptor, which interacts molecularly and genetically with two other transmembrane, EGF-containing proteins of the *Notch* group: Serrate and Delta. The other known members of the *Notch* group are *deltex, Enhancer of split*, and *mastermind*. The *deltex* gene seems to code for a cytoplasmic protein, while *mastermind* and *Enhancer of split* encode nuclear proteins. Recent evidence indicates that at least some members of the *Notch* group are present in the human genome and may also be involved in cell fate decisions.

The molecular mechanisms that control how neuronal growth cones find and recognize their correct targets during development continue to be the focus of Investigator Corey S. Goodman, Ph.D. (University of California, Berkeley) and his colleagues. Molecular genetic approaches in *Drosophila* are used to

address these issues. Based on their increasing knowledge of the cell biology and the availability of specific probes, this laboratory has undertaken a series of large-scale, systematic mutant screens in order to identify genes that control growth cone guidance and target recognition in the *Drosophila* embryo. Over the past year, many new genes have been identified, including genes that 1) control the guidance of growth cones either toward or away from the midline either to enter or avoid the commissures of the central nervous system, 2) control the guidance of growth cones along longitudinal axon pathways of the central nervous system, and 3) control the ability of motoneuron growth cones to find and recognize their appropriate target muscles. Many of these genes are likely to encode important components of mechanisms that impart specificity on the events of pathway and target recognition. Several of these new genes, including *commissureless* and *roundabout*, are under molecular investigation.

The developing *Drosophila* retina provides one of the few systems where the molecular mechanisms of short-range inductive interactions can be studied at the level of individual cells. The fate of a cell within the retina appears to be governed by the specific combination of signals received by that cell from its immediate neighbors. Research in the laboratory of Investigator Gerald M. Rubin, Ph.D. (University of California, Berkeley) is aimed at gaining an understanding of how such signals are generated, sensed, and responded to by cells in the developing retina. During the past year the laboratory has used a novel genetic approach to identify several of the components that act downstream of a transmembrane protein-tyrosine kinase receptor that transduces a signal essential for the determination of one particular photoreceptor cell type.

Associate Investigator S. Lawrence Zipursky, Ph.D. (University of California, Los Angeles) and his colleagues also have been studying visual system development in the fruit fly. This model has allowed them to address several fundamental questions in development. How do cells communicate during development to regulate that the right cell forms in the right place? What mechanisms control the rate of cellular proliferation? And finally, how do neurons form precisely arranged networks of interconnected cells? Through the molecular and developmental analysis of mutants defective in visual system development, this laboratory has gained insight into the molecular mechanisms underlying some of these processes.

The overall objective of the research of Assistant Investigator Hermann Steller, Ph.D. (Massachusetts Institute of Technology) and his colleagues is to understand how functional neuronal circuits are established and maintained during development. Their efforts also are focused primarily on visual system development in *Drosophila*, where these processes can be studied at the level of individual cell types. They have isolated mutations that affect the generation of specific neurons, the formation of proper neuronal connections, and the elimination of cells through programmed cell death. Genes corresponding to some of these mutants have been cloned and are being analyzed.

The main goal of the laboratory of Associate Investigator Charles S. Zuker, Ph.D. (University of California, San Diego) is to elucidate mechanisms used for signal transduction in sensory systems. In particular, the work focuses on a molecular genetic dissection of visual transduction and mechanotransduction. Several molecules playing critical roles in the *in vivo* regulation of the phototransduction cascade process have been characterized and include members of the family of immunosuppressing binding proteins, two arrestin homologues, and enzymes involved in metabolism of the intracellular messenger inositol trisphospate. Work on mechanoreceptors is aimed at understanding how the mechanical senses—for instance, touch, hearing, and balance—work at a molecular level. *Drosophila* bears an abundance of mechanosensory organs, some of which show physiological similarities to mechanoreceptors in the vertebrate inner ear. The laboratory has isolated several mutations that affect the development or gross structure of the *Drosophila* external adult mechanosensory organs and some in which the organs are all present and properly formed but may have abnormal physiology.

Research of the laboratory of Associate Investigator Gary Struhl, Ph.D. (Columbia University) is directed toward determining the molecular nature and mode of action of spatial information responsible for organizing cell and body patterns in *Drosophila*. Several potential signaling molecules have been identified and their functions assessed by altering their structures or patterns of expression. These studies have established that most aspects of anteroposterior body pattern are specified in early embryos by the graded distributions of two transcription factors, *bicoid* and *hunchback* protein. They have also implicated the secreted *wingless* protein as a morphogen controlling dorsoventral pattern in the developing limbs.

ION CHANNELS MEDIATING ELECTROCHEMICAL SIGNALING IN VERTEBRATE NEURONS

PAUL R. ADAMS, PH.D., *Investigator*

Nerve cells express a panoply of plasma membrane ion channels that mediate electrical and chemical signaling on a variety of time scales. These channels open and close in response to changes in local membrane voltage or chemical environment. Ion flow through the open channels then causes further changes in local voltage or chemistry. These changes can spread to adjacent cell regions, eventually leading to coordinated electrical responses, such as propagating action potentials or synaptic transmission. Dr. Adams's laboratory investigates how various types of ion channel contribute to these electrical responses and how cell geometry influences signal spread. This is achieved with a combination of electrophysiological techniques (such as patch-clamp recording) and specialized light microscopy (such as calcium imaging and three-dimensional reconstruction).

Nerve Cells in Three Dimensions

The electrical activity of neurons is played against a framework of passive membrane capacity distributed in an intricate three-dimensional array. Embedded within this array is a network of ion channels fronting complex cytoplasmic diffusion pathways. Dr. Adams's laboratory has combined confocal microscopy of sympathetic ganglion cells, hippocampal neurons, and lateral geniculate relay cells with custom voxel-based ray-tracing software ("Volvis," developed in collaboration with Dr. Ari Kaufman's laboratory in Stony Brook).

Use of various fluorescent probes allows different aspects of the cell to be visualized. Negative staining with fluorescein dextrans permits the outer cell surface to be seen, as the cell makes a hole in the fluorescent medium, which can then be reversed on a graphics workstation. Conversely, intracellular staining with lucifer yellow reveals the inner cell surface and details of intracellular structures. Cell membranes are visualized with nile red, synapses with mitochondrial stains, and intracellular calcium with FLUO 3. Rendering algorithms are then used to provide a vivid three-dimensional view from any desired aspect.

Any relevant geometrical parameter is easily extracted from these large three-dimensional data sets. For example, cell surface area measurement is compared with electrical capacity measurements to explain the enigma that the specific capacitance of the neuronal membranes is often considerably greater than that allowed by its lipid bilayer structure.

Because living cells move, three-dimensional visualization is also necessary to explore quantitatively optical signals from small structures like spines that are close to the resolution limits of light microscopy.

Calcium Imaging

In previous work the laboratory characterized rapid cytoplasmic calcium redistribution in ganglion cells following brief voltage-clamp depolarizations by using a line-scanning technique. This method is rather insensitive to spatial inhomogeneities in calcium dynamics. Two-dimensional confocal imaging of calcium signals across the entire cell has now been achieved at five frames per second, with improved-resolution optical sectioning and calcium sensitivity. Work in the laboratory had previously shown that a major problem with rapid calcium imaging is dye redistribution, which tends to collapse calcium gradients. This is being solved by using lower dye concentrations and slowly diffusing dyes. Calcium entry seems to be uniform along the cell membrane (after making allowance for surface-to-volume ratio effects). Detection of true calcium "hotspots" or "domains," if they exist, will require further improvement in imaging speed.

M-Current Regulation

Many neurons express M channels, which are non-inactivating voltage-dependent potassium channels controlled by various neurotransmitters acting through an unknown G protein. These channels limit action-potential firing frequency unless turned off by impinging synaptic activity. The laboratory has continued to examine mechanisms underlying this synaptic regulation of M current. Fast-flow techniques were used to establish the time course of transmitter action. After a latency of 200 milliseconds, M-channel closure peaks ~2 seconds after very brief transmitter applications, and then channels reopen over the next minute. At the termination of longer pulses, supranormal channel reopening is seen, leading to overrecovery. These observations suggest that the channels can operate in three distinct gating modes. These can be directly observed by using on-cell single-channel recording.

The roles of calcium and arachidonic acid metabolism in M-channel inhibition and overrecovery have been further investigated. It had been shown that M-channel activity is optimal at physiological levels (80 nM), declining at higher or lower levels. It has

now been observed that intracellular calcium levels also affect the sensitivity of the channels to agonist. The effects of muscarine and LH-RH (luteinizing hormone-releasing hormone) are much smaller when intracellular calcium is completely absent, suggesting that calcium-dependent proteins participate in the transduction pathway.

Arachidonic acid increases M current, and phospholipase A_2 inhibitors, such as quinacrine and bromophenacyl bromide, prevent both overrecovery and calcium-induced enhancement of M current. Furthermore, 12-lipoxygenase pathway metabolites mimic calcium enhancement and overrecovery, while the 12-lipoxygenase pathway inhibitor baicalein inhibits all these effects. Thus arachidonic acid metabolites are plausible mediators of both calcium enhancement and overrecovery. However, these pathways do not appear to be involved in the primary inhibition elicited by agonists.

Dr. Adams is also Professor of Neurobiology and Behavior and of Pharmacological Sciences at the State University of New York at Stony Brook.

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MOLECULAR MECHANISMS OF VOLTAGE-GATED ION CHANNEL FUNCTION

Richard W. Aldrich, Ph.D., *Associate Investigator*

Voltage-gated ion channels are the molecular elements that underlie electrical signaling in excitable and nonexcitable cells. Among the important physiological processes in which they are involved are information processing and transmission in the nervous system, neuronal plasticity, initiation and regulation of the heartbeat, and muscle excitation. The voltage sensitivity and time course of opening and closing of these channels in response to changes in membrane potential determine how a given cell generates electrical activity and responds to signals from other cells. The long-term goal of research in Dr. Aldrich's laboratory is to understand the molecular mechanisms of ion channel function.

Dr. Aldrich and his colleagues have studied gating processes in voltage-gated potassium channels from the *Shaker* family. *Shaker* channels have been used as a model system for voltage-dependent channel gating for several reasons. The subunits are smaller and therefore more readily manipulable than voltage-dependent sodium or calcium channels. The wide diversity of structural and physiological properties of channels in the *Shaker* superfamily can facilitate the identification of structural regions of particular functional significance.

The general strategy has been to combine single-channel, macroscopic, and gating current measurements with alterations of channel structure by *in vitro* mutagenesis to define amino acid residues that are involved in specific conformational changes and to determine the biophysical mechanisms involved in these changes. The research has focused on three different gating processes: 1) inactivation mediated by the amino-terminal (N-type inactivation), 2) a separate inactivation process mediated in part by residues in the sixth membrane-spanning region (C-type inactivation), and 3) voltage-dependent channel activation.

Mechanisms of N-Type Inactivation

Shaker channels become inactive quite rapidly after opening, within less than a millisecond in some *Shaker* variants. Previous studies by Drs. Aldrich, Toshinori Hoshi, and William Zagotta provided evidence for a mechanism whereby an intracellular portion of the channel, consisting of amino acid residues near the amino terminal, effects inactivation, occluding the internal mouth of the channel. A crucial piece of evidence for this mechanism was that a synthetic peptide with the sequence of the amino-terminal domain was able to restore inactivation in channels that did not inactivate because of mutations in the amino-terminal region.

Dr. Ruth Murrell-Lagnado and Dr. Aldrich have investigated the determinants of the interaction between the inactivation-inducing peptides and the

channel mouth. They have found that the net charge on the carboxyl-terminal half of the peptide is quite important for determining the rate of binding, whereas the stability of the bound (inactivated) state is predominately determined by hydrophobic interactions between residues in the amino-terminal half of the peptide and its binding site on the channel.

All *Shaker* potassium channels contain a consensus cAMP-dependent phosphorylation site near the carboxyl terminal. In addition, the *Shaker* D variant has a potential cAMP phosphorylation site in the amino-terminal inactivation particle region. To test if these sites are important for regulating the properties of the channels, Drs. Peter Drain, Adrienne Dubin, and Aldrich applied bovine alkaline phosphatase (BAP) and cAMP-dependent protein kinase (PKA), with appropriate substrates and cofactors, to the internal face of inside-out patches from oocytes expressing *Shaker* D. An increase in current and a decrease in the time constant of macroscopic inactivation upon application of BAP could often be reversed by PKA. These results demonstrate a potential mechanism for regulation of neuronal activity by modulation of potassium channel inactivation rates. (The project described above was supported in part by a grant from the National Institutes of Health.)

Mechanisms of C-Type Inactivation

The rate of C-type inactivation can vary by two orders of magnitude among different carboxyl-terminal *Shaker* variants. In many previous studies of *Shaker* channel inactivation, the distinction between the two types of inactivation has not been made clear. However, because N-type inactivation can be eliminated by mutations in the amino-terminal domain, C-type inactivation can be studied in isolation. Using amino-terminal mutants, Drs. Hoshi, Zagotta, and Aldrich constructed chimeras from *Shaker* variants with C-inactivation rates differing by two orders of magnitude. These and subsequent point mutations allowed them to localize, in the sixth membrane-spanning region, a single–amino acid difference that was responsible for the large difference in rates between these two variants. (The project described above was supported in part by a grant from the National Institutes of Health.)

Because the substitution that caused the large difference in C-type inactivation rates was a change between an alanine and a valine, Drs. Hoshi, Zagotta, and Aldrich examined the effects of substitution of other nonpolar amino acids at this position. Channels with a glycine inactivated slowly, much like the alanine-containing channels, whereas chan-

nels with an isoleucine inactivated with an intermediate rate. Surprisingly, these mutations also changed the single-channel conductance, with valine- and isoleucine-containing channels having about a 1.5 and 2 times larger conductance, respectively, than alanine- or glycine-containing channels. Paul Zei, Dr. Hoshi, and Dr. Aldrich found that the changes in conductance do not reflect an alteration in ion selectivity of potassium relative to sodium, rubidium, or ammonium. Drs. Jose Lopez-Barneo, Stefan Heinemann, Hoshi, and Aldrich have found that the C-type inactivation rate can be markedly influenced by external cations in a manner consistent with the idea that inactivation is impeded when the channel is occupied by permeant ions. (The project described above was supported in part by a grant from the National Institute of Mental Health's Silvio Conte Center for Neuroscience Research.)

Mechanisms of Voltage-Dependent Activation

The ability to eliminate N-type inactivation and to slow C-type inactivation by mutations allows the study of single–*Shaker*-channel activation in relative isolation from inactivation. Dr. Hoshi, Dr. Zagotta, Jeremy Dittman, and Dr. Aldrich have performed a detailed study of the single-channel, macroscopic, and gating currents in these channels to determine the minimum number of states required, the transition rates between them, and the voltage dependence of these rates. The results have been used to develop an understanding of wild-type channel activation that will provide a basis for interpreting the results of mutagenesis. (The project described above was supported in part by grants from the National Institutes of Health and the National Institute of Mental Health's Silvio Conte Center for Neuroscience Research.)

Dr. Aldrich, Dr. Catherine Smith-Maxwell, Dr. Zagotta, Max Kanevsky, Jennifer Ledwell, Michael Root, and Melinda Przetak have generated and studied mutations in regions shown to be involved in voltage-dependent activation. A particularly interesting mutation is one in which a leucine residue in the cytoplasmic loop between the fourth and fifth membrane-spanning regions is changed to a tyrosine. Alanine, phenylalanine, and tyrosine substitutions at this position result in shifts in the conductance-voltage relationship to more positive potentials. The tyrosine substitution, however, drastically changes the macroscopic activation kinetics, in addition to the shift in the voltage dependence. The wild-type channels activate much faster than the tyrosine mutants. The importance of the hydroxyl group may lie in its ability to hydrogen bond to solvents.

Dr. Aldrich is also Associate Professor of Molecular and Cellular Physiology at the Stanford University School of Medicine.

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CELLULAR AND MOLECULAR MECHANISMS OF NEURAL CREST DEVELOPMENT

DAVID J. ANDERSON, PH.D., *Assistant Investigator*

Regulation and Function of *MASH* Genes

To begin to identify nuclear regulatory factors that control the commitment of neural crest cells to various sublineages, Dr. Anderson and his colleagues have cloned vertebrate homologues of *Drosophila* genes involved in neural development. Two such genes are *MASH* (mammalian *achaete-scute* homologous) *1* and *2*, homologues of *Drosophila achaete-scute*. These genes encode members of the basic helix-loop-helix (bHLH) family of transcription factors, which includes the myogenic determination gene *MyoD*. *MASH1* and *MASH2* are >80% identical to their *Drosophila* counterparts within the bHLH domain, but diverge outside this region.

Analysis of the pattern of *MASH1* expression using a monoclonal antibody has revealed that this gene, like its *Drosophila* counterpart, is expressed specifically and transiently in spatially restricted subsets of precursor cells within the developing nervous system. Together with similar data obtained by others for mammalian and *Drosophila MyoD* (*nautilus, nau*), these results suggest that genes encoding bHLH proteins may constitute an evolutionarily conserved family of cell-type determination genes, of which *MASH1* is the first neural-specific member to be identified in vertebrates.

The deduced amino acid sequence of the MASH proteins predicts that they should function as transcription factors. To provide experimental evidence in support of this prediction, recombinant MASH proteins were expressed in different systems and their DNA-binding and transcriptional regulatory activities assessed. Both MASH1 and MASH2 were found to bind to an E box, the consensus binding site for all bHLH proteins, but only as hetero-oligomers with the ubiquitously expressed bHLH protein E2A.

Surprisingly, MASH1 and MASH2 also activated transcription of a muscle-specific gene, muscle creatine kinase (*MCK*), which contains an E box and is thought to be a target of MyoD. Unlike *MyoD*, however, the *MASH* genes were unable to activate the full myogenic program in transfected C3H-10T½ cells, even if the *MyoD* basic region was substituted for the *MASH1* basic region. Although MCK is unlikely to be a physiological target of the *MASH* genes, these data indicate that MASH1 and MASH2 are indeed transcription factors and identify a biochemical assay system in which structure-function relationships in these proteins can be explored. Studies are now under way to identify authentic targets of MASH proteins using a variety of experimental strategies.

To identify a culture system in which the regulation and function of *MASH* genes can be studied, Dr. Anderson's laboratory examined their expression in mouse P19 embryonal carcinoma cells. In such cells, *MASH1* mRNA and protein are initially undetectable, but are expressed upon induction of neuronal differentiation by retinoic acid. Furthermore, MASH1 protein expression precedes and then overlaps expression of neuronal markers such as NCAM (neural cell adhesion molecule). However, MASH1 is not detected in differentiated P19 neurons, suggesting that (as is the case *in vivo*), its expression

during neurogenesis is transient. Although MASH1 expression precedes neuronal differentiation, transfection of *MASH1* expression constructs into P19 cells failed to induce neuronal differentiation in the absence of retinoic acid. Nevertheless, the exogenously expressed MASH1 protein is able to bind to DNA in nuclear extracts from the transfected cells. These data suggest that MASH1, unlike its muscle relative MyoD, is not sufficient for neurogenesis.

Unexpectedly, *MASH2* displayed a pattern of expression in P19 cells complementary to that of *MASH1*. Low levels of *MASH2* mRNA were detectable in uninduced cells and declined following retinoic acid treatment in parallel with the onset of neuronal differentiation. This suggests that the two *MASH* genes, despite their near sequence identity in the bHLH region, play very different roles in mammalian development. Consistent with this idea, preliminary *in situ* hybridization analysis revealed that the highest levels of *MASH2* expression are found in the ectoplacental cone, a primitive extraembryonic membrane. Furthermore, uniform low levels of *MASH2* mRNA are detected throughout the embryo.

Gene mapping studies performed in collaboration with Dr. Nancy Jenkins of the National Cancer Institute have indicated that *MASH1* maps to murine chromosome 10 and *MASH2* to chromosome 7. This is to be contrasted with the *Drosophila achaete-scute* genes, which exist in a tightly linked chromosomal complex. Each *MASH* gene is closely linked to an insulin-like growth factor (IGF) gene; *MASH1* to IGF-1 and *MASH2* to IGF-2. This suggests that they may represent a duplicated chromosomal region. Furthermore, *MASH2* is located in a region of chromosome 7 that is known to display the phenomenon of imprinting. The significance of this finding remains to be explored.

To examine the biological function of the *MASH* genes *in vivo*, loss-of-function mutations are being generated by the technique of homologous recombination in embryonic stem (ES) cells. Currently several ES lines containing a "knockout" mutation in the *MASH1* gene have been isolated. These lines have been injected into mouse blastocyst-stage embryos, generating chimeric mice. If this chimerism proves to extend to the germline, then homozygous mutant animals will result. The effects of this mutation on embryonic nervous system development will then be explored in detail.

Control of Cell Fate by Glucocorticoids in the Sympathoadrenal Lineage

In the sympathoadrenal lineage, two aspects of chromaffin cell development are dependent upon glucocorticoids: the inhibition of neuronal differentiation and the induction of the epinephrine-synthesizing enzyme phenylethanolamine *N*-methyltransferase (PNMT). In the developing embryo, these two aspects are temporally separated. This raises the question of how the same inducing signal can control two different biological responses at different stages of development.

Using cultures of purified chromaffin precursor cells, Dr. Anderson and his student Arie Michelsohn have shown that both effects of glucocorticoid appear to be mediated by the same type of receptor. The temporal separation appears to be due to a cell-autonomous clock that changes the way the cells respond to the steroid, according to an intrinsic developmental schedule. This two-step pathway appears to provide a "checkpoint" mechanism that ensures that only chromaffin cells, and not sympathetic neurons, will be able to synthesize epinephrine. (This project was supported by a grant from the National Institutes of Health.)

Dr. Anderson is also Associate Professor of Biology at the California Institute of Technology and Adjunct Assistant Professor of Anatomy and Cell Biology at the University of Southern California School of Medicine.

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CELL FATE CHOICES IN DEVELOPMENT

SPYRIDON ARTAVANIS-TSAKONAS, PH.D., *Investigator*

Dr. Artavanis-Tsakonas and his collaborators have been investigating the mechanisms of cell communication that control the choice of cellular fates in *Drosophila* development. They are particularly interested in how these rules apply to the nervous system and have been studying the molecular biology and genetics of a group of genes that are involved in cell fate choices in neural development.

Genes Controlling Cell Fate Choices

A variety of studies have indicated that the ability of a cell to choose between an epidermal and neuronal developmental pathway depends on interactions between neighboring cells. In *Drosophila*, the *Notch* locus, which was shown several years ago by the Artavanis-Tsakonas group to code for a transmembrane protein with homology to the epidermal growth factor (EGF), is known to play a central role in this process. Phenotypic analysis of many *Notch* alleles, as well as *in situ* hybridization and antibody localization experiments, have shown that in addition to neuronal differentiation, *Notch* controls the differentiation of numerous tissues. The accumulated evidence indicates that the *Notch* gene product functions in a rather general signal transduction mechanism required for many different types of cell fate decisions. Many if not all of the regulative events required throughout development for the correct differentiation of neighboring precursor cells into distinct developmental paths appear to depend on *Notch*-mediated signals.

The notion that *Notch* plays a role in a cell communication mechanism implies the existence of several interacting components. In an attempt to understand the biochemical nature of *Notch* and identify interacting partners, much of the research effort of the Artavanis-Tsakonas laboratory is addressed to the dissection of the genetic circuitry in which *Notch* is integrated. Based on several criteria, a group of interacting genes have already been identified and were operationally termed the "*Notch* group." So far this group consists of *Notch*, *Delta*, *mastermind*, *Enhancer of split*, *deltex*, and *Serrate*. The gene products of the *Notch* group were shown to code for nuclear and cytoplasmic as well as cell surface elements. During the past year the Artavanis-Tsakonas group has been studying the molecular and genetic relationships among the members of the *Notch* group while continuing the search for additional elements involved in *Notch*-mediated cell communication.

Notch, Delta, and *Serrate*

Apart from *Notch*, two other *Notch* group members were shown to code for cell surface elements: *Delta* and *Serrate*. Both code for transmembrane proteins with extracellular domains displaying homology to EGF, and both were shown to interact with *Notch* molecularly as well as genetically. Using a cell aggregation assay, the Artavanis-Tsakonas group demonstrated that *Notch* is capable of interacting with *Delta* and *Serrate* via their extracellular domains.

Through an extensive deletion mutagenesis of the extracellular domain of *Notch*, the group further found that of the 36 EGF repeats of *Notch*, only two (11 and 12) are both necessary and sufficient to mediate interactions with *Delta* and *Serrate*. Fur-

thermore, this binding ability is conserved in the corresponding two repeats from the *Xenopus Notch* homologue. These results raise the possibility that *Notch* may act as a multifunctional receptor whose 36 EGF repeats form a tandem array of discrete ligand-binding units, each of which may potentially interact with several different proteins during development.

Recent efforts have been directed toward addressing the *in vivo* relevance of this system by introducing various *Notch* deletion constructs into flies via P-element–mediated transformation and then studying their biological activity. All constructs were placed under the control of two promoters: the regular *Notch* promoter, in order to be able to study the effects of mutant *Notch* proteins expressed at the appropriate times and places during development, and the hsp70 promoter, in order to look at the effects of overexpression of these constructs in tissues where *Notch* is not normally expressed.

Examination of cell aggregates expressing *Notch* and *Delta* showed that the proteins are colocalized at regions of cell contact and in vesicles within the *Notch*-expressing cells. Similar vesicles are also observed in imaginal discs stained for *Notch* and examined by light or electron microscopy. The internalization of *Delta* into *Notch*-expressing cells was further investigated by performing live-cell antibody stainings on the S2 cell lines. Within 5 minutes of the mixing of the two cell lines, *Notch* and *Delta* become colocalized at adhesion interfaces, and within 15 minutes both proteins are internalized into the *Notch*-expressing cell. Identical internalization occurs in S2 cells, which express only the extracellular portion of *Notch*, indicating that this process does not require the *Notch* intracellular domain.

The notion that *Notch* and *Delta* may engage in a receptor-ligand relationship is supported by genetic mosaic experiments in Dr. Pat Simpson's laboratory. It was found that cells mutant for *Notch*, but not *Delta*, display cell autonomy.

Genetic Interactions Involving *Notch*

To identify other genes involved in *Notch* function, Dr. Artavanis-Tsakonas and his co-workers have initiated two genetic screens for loci that interact with *Notch* in a dosage-dependent manner. The first screen is based on the observation that N^{ts1}/fa^{g2} transheterozygous females exhibit a strong temperature sensitivity with respect to their fa^{g2} eye phenotype yet remain fully viable and fertile at all temperatures from 18°C to 30°C. So far ~30 mutant candidates have been obtained from among ~30,000 N^{ts1}/fa^{g2} female progeny. By searching for loci that display haplo-insufficient enhancement of a sensitized *Notch* mutant phenotype, it will be possible to identify genes not previously associated with *Notch* function as a result of their embryonic lethality and/or lack of a neurogenic phenotype in the null state.

The second genetic screen was designed to isolate enhancers of *notchoid* (*nd*). This is a hypomorphic *Notch* allele that affects wing morphology, has been shown to interact with several *Notch* group members, and is associated with a missense mutation in the intracellular domain of the protein. A screening of 120,000 flies for modification of the *nd* wing phenotype yielded 140 mutants with modified wings. The complementation analysis carried out so far has revealed approximately 10 complementation groups. As expected, some are members of the *Notch* group as defined now, but more importantly, new genes whose relationship with *Notch* was hitherto unknown have also been detected.

The *Notch* Group in Mammals

In an attempt to examine the analogies that may exist between the invertebrate experimental model and vertebrates, as well as to take advantage of the sophisticated cell culture systems that the vertebrate model offers, the Artavanis-Tsakonas group started to isolate and study the human counterparts of the *Notch* group of genes. So far, human counterparts of two *Notch* group members have been isolated: *Notch* and *groucho* (the latter, a gene of the *Enhancer of split* complex).

While the fly appears to have a single *Notch* gene, vertebrates have at least two different genes encoding *Notch*-like proteins. In humans the Artavanis-Tsakonas group has demonstrated the existence of two *Notch* homologues. Interestingly, other workers have associated rearrangements of one of the human *Notch*-like genes with a particular neoplasm, suggesting an important developmental role for the human *Notch*.

The *Drosophila m9/10*, or *groucho*, gene is characterized by the presence of a tandem array of so-called WD-40 repeats at its carboxyl-terminal half. Such a motif was first identified in the β subunit of transducin, but has since been found in a growing number of proteins associated with diverse cellular functions. By screening a cDNA library of fetal human brain, four *groucho* homologous genes encoding a family of proteins have been isolated and designated *TLE* (transducin-like *Enhancer of split*). The *TLE* genes were shown to be expressed differentially and to encode nuclear proteins. This attri-

bute is consistent with the presence of sequence motifs previously associated with nuclear functions. The existence of more than a single *TLE* and *Notch* gene suggests that structural redundancy may be a feature of the human counterparts of the developmentally important genes of the *Drosophila Notch* group.

The extraordinary structural conservation among the *Drosophila* and human gene products implies that the biochemical mechanisms involving *Notch* and *Enhancer of split* may also be conserved across species boundaries. However, the identification of multigene families suggests that gene duplication events have created a more complex situation in mammals. If the *Notch* group role has been conserved from flies to vertebrates, then a general and pleiotropic pathway involved in controlling many aspects of mammalian cell fate may have been identified.

Dr. Artavanis-Tsakonas is also Professor of Cell Biology and Biology at the Boyer Center for Molecular Medicine at Yale University School of Medicine.

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THE MOLECULAR BIOLOGY OF SMELL: GENES ENCODING RECEPTORS AND CHANNELS IN THE NOSE

Richard Axel, M.D., *Investigator*

Environmental stimuli are recognized by sensory neurons, and this information is transmitted to the brain, where it is decoded to provide an internal representation of the external world. The olfactory sensory system of vertebrates can recognize and discriminate a large number of odorants of diverse molecular structure. For example, humans are capable of distinguishing thousands of distinct odors. Although odorants often exhibit widely different structures, subtle changes in molecular structure can lead to striking differences in perceived odor. How do olfactory sensory neurons recognize the vast array of molecular structures that are defined as odorants? The detection of distinct odorants presumably results from the association of odorous ligands with specific receptors on olfactory neurons. Discrimination among distinct odors will then require that the brain distinguish which receptors have been activated.

In initial experiments to define the logic underlying olfactory perception, Dr. Axel and Dr. Linda Buck identified and cloned a large family of genes that are likely to encode odorant receptors in the rat. This multigene family encodes seven transmembrane domain receptors and belongs to the superfamily of G protein–coupled receptors. The size of the gene family in mammals (perhaps as large as 1,000 genes) suggests that olfactory perception employs a large number of receptors, each capable of interacting with one or a small number of odorous ligands. The interaction of odorous ligands with a large number of receptors on olfactory sensory neurons provides a mechanism for the specific recognition of a diverse array of odors.

Isolation and Sequence of Catfish Olfactory Receptor cDNAs

In subsequent experiments to discern the mechanism of processing of olfactory information, Dr. Axel and his colleagues have asked whether cells expressing a specific odorant receptor (and therefore responsive to specific odors) are spatially segregated in the olfactory epithelium. The channel catfish was chosen as a model system for two major reasons: first, the repertoire of putative odorant receptors is numerically far simpler than in mammals, and second, the catfish olfactory epithelium is organized in a simple, regularly repeating anatomic structure. It is therefore possible to examine the patterns of receptor expression of a significant fraction of the repertoire with a relatively small number of receptor probes.

The isolation of cDNA clones encoding putative olfactory receptors from catfish sensory neurons was

based upon the assumption that these receptors will share considerable homology to the family of rat olfactory receptors. The deduced amino acid sequences of six catfish olfactory cDNAs again define a family of proteins that share sequence and structural properties with the seven-transmembrane-domain superfamily of neurotransmitter and hormone receptors. Some sequence motifs are conserved in the olfactory receptors identified in both fish and mammals, whereas other motifs appear specific for fish receptors. Overall, the individual fish receptors exhibit 40–60% amino acid homology to one another and 30–40% homology with representative rat sequences.

If the odorant receptor repertoire reflects the range of detectable odors, fish might be expected to possess a far smaller number of odorant receptors than mammals. Extensive PCR (polymerase chain reaction) and cloning experiments identify only 20 different receptor genes in the catfish, whereas similar experiments in mammals identify several hundred genes. Southern blotting of DNA from a single catfish with three divergent cDNA probes confirms this suggestion. Thus the number of olfactory receptor cDNA genes present within the catfish genome is significantly smaller than that observed in mammalian DNA, as a consequence of the smaller size and smaller number of subfamilies. The concordance between the range of detectable odors in different vertebrates and the size of the receptor repertoire is consistent with a mechanism of odor recognition in which individual odorant receptors associate with only one or a few odorants.

Spatial Segregation and the Processing of Olfactory Information

Although these studies suggest a mechanism by which odors are recognized, discrimination among the vast array of odors requires that the brain discern which of the numerous receptors have been activated. By analogy with other sensory systems, it has been suggested that this is accomplished by the identification of which neurons in the olfactory epithelium are activated by different odorants. Such identification could result from spatial segregation of functional classes of neurons in the epithelium and from the spatially defined projections of sensory neurons to the olfactory bulb. In the simplest form of this model, each olfactory neuron would express only one type of receptor, and each cell bearing the same receptor would project to the same glomerulus in the olfactory bulb. The numerical simplicity of the repertoire of receptors in the fish has allowed estimates of the diversity of receptor expression in a

single neuron. Analysis of *in situ* hybridization to olfactory sensory epithelium with three distinct receptor probes provides preliminary evidence that individual neurons are likely to express one or a small number of receptors.

If the identity of a given neuron is identified by the nature of the receptor it expresses, then the identity of a given odorant can be defined by the cells it activates. How then does the brain discern which neurons have been activated by a specific odorant? In one model, neurons expressing a given receptor are spatially localized within the epithelium and project to one or a few spatially segregated glomeruli within the olfactory bulb. In a second model, neurons expressing a given receptor may exhibit no spatial order and may be randomly distributed throughout the epithelium, while their axons project to one or a few glomeruli with spatially defined loci within the bulb. In either instance, exposure to a given odorant would result in the stimulation of a spatially restricted set of glomeruli such that individual odorants would be associated with specific topographic patterns of activity within the bulb. Distinguishing among these other models would require an examination of the pattern of receptor expression in the neurons of the olfactory epithelium and the pattern of projections these neurons exhibit in the olfactory bulb.

Dr. Axel and his colleagues have used probes representative of three distinct receptor subfamilies encompassing about 20 different genes and find no evidence for the restricted spatial localization of neurons expressing specific odorant receptors within the olfactory epithelium, with no segregation along anterior-posterior, dorsal-ventral, or medial-lateral axes. Furthermore, three-dimensional reconstruction techniques fail to demonstrate more-subtle patterns of receptor expression. Neurons expressing specific receptors therefore appear to be distributed randomly within the olfactory epithelium. Thus specific odors are unlikely to elicit defined spatial patterns of activity within the olfactory epithelium. Rather, dispersed olfactory neurons with common receptors are likely to converge on common glomeruli in the olfactory bulb.

These data contrast with electrophysiological experiments in higher vertebrates that suggest the spatial segregation of functionally discrete classes of neurons in olfactory epithelium, which is maintained in the projections to the bulb. The availability of molecular probes for mammalian receptors should now permit a direct analysis of the patterns of expression of odorant receptors in mammals and may provide insight into the strategies employed in their more complex olfactory systems.

Dr. Axel is also Higgins Professor of Biochemistry and Molecular Biophysics and Professor of Pathology at Columbia University College of Physicians and Surgeons.

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ACTIVATION AND REGULATION OF ION CHANNELS

David P. Corey, Ph.D., *Associate Investigator*

Dr. Corey's laboratory is interested in the regulation of membrane permeability in neurons, which underlies such processes as sensory transduction, membrane excitability, and synaptic transmission. The broad goal is to understand the ion channel proteins that mediate permeability, the mechanism of their activation, and the processes of their expression and control.

The special interest of Dr. Corey's group is ion channels that are activated by direct mechanical stress on the channel protein. Such channels may underlie a variety of mechanical sensitivities in different tissues. In Dr. Corey's laboratory, they are being studied specifically in hair cells, the mechanically sensitive receptor cells of the inner ear that transduce sound into an electrical signal. These cells bear a bundle of 50–200 cilia on their apical surface, and deflection of the bundle by sound opens ion channels in the tips, allowing ionic current to enter the cell. A long-standing theory for transduction is that deflection of the bundle stretches fine filaments, or "tip links," that extend between the tips of the cilia, and that these pull directly on the ion channels to open them. Last year, Dr. Corey's group was able to show that tip links do pull on the channels, which helps to confirm the theory.

Physiological Limits to Adaptation

There is also an adaptation mechanism that follows displacements. During a maintained positive displacement, the transduction current declines over tens of milliseconds, which seems to result from a relaxation of the tension on channels. Similarly, a displacement that allows channels to close

activates a retensioning mechanism that reopens channels. Together these act to keep a constant proportion of channels open. More specifically, the adjustment appears to come about by a movement of one end of the elastic element that conveys tension to the channel. Work from several laboratories has led to the thought that some motor element continually tries to increase tension on the channels, but slips if the tension becomes too great.

This year Dr. Corey's laboratory has found that this adaptation mechanism only works over a limited range. For small deflections (up to ~ 0.7 μm in either direction, as measured at the bundle's tip), the motor successfully adjusts the tension to restore the resting level. For larger deflections, however, the tension adjustment is incomplete. The motor seems to hit a limit of some sort, beyond which it cannot move.

Structural Basis of Adaptation

The tip-link hypothesis can be combined with the adaptation model, by imagining that the motor element moves the upper end of the tip link along the side of a stereocilium. The structural correlate of adaptation would thus be the movement of the attachment point. In the past two years, Dr. Corey and his colleagues have been testing this view, with cells fixed before and after adaptation. The osmiophilic densities marking the tip-link attachments were observed with electron microscopy. In a quantitative analysis of micrographs, the densities were found to move along the sides of stereocilia in the direction predicted. When tip links were cut, relieving tension on the densities, they climbed along the stereocilia. On the basis of these observations,

it seems likely that adaptation is mediated by movement of the tip-link attachments along the stereocilia.

Based on the physiological limits to adaptation, however, the expected movement of the densities is also limited. In fact, the average movement measured from thousands of electron micrographs was no more than ~50–75 nm, even for very large deflections of the bundles. This is in good agreement with the prediction from the physiological limit. Still unclear is what sort of microscopic structure is responsible. Is it like a fence or a tether?

Hyperkalemic Periodic Paralysis

In a related project Dr. Stephen Cannon in Dr. Corey's laboratory has been working on the molecular basis of an inherited muscle disease, hyperkalemic periodic paralysis (HPP), which causes weakness or paralysis when serum potassium is raised. Last year Dr. James Gusella's laboratory (Massachusetts General Hospital) found that the gene for the sodium channel α subunit was coinherited with HPP. Dr. Cannon then studied muscle from an affected patient and found that the sodium channels were themselves defective: with high extracellular potassium, they occasionally fail to inactivate.

Still remaining were three important questions: Is there a genetic defect in the α subunit? Can such a defect account for the inactivation failure observed in HPP muscle? Does the inactivation failure fully account for the pathology?

This year several groups were able to identify single nucleotide substitutions in the α subunit from patients with HPP. There are different mutations in different families, indicating that the disease arises spontaneously. Thus defects in the gene coinherit with the disease.

Dr. Cannon then inserted two of these mutations into the sodium channel α-subunit gene from the rat. As expected, the mutated channels, when expressed in a human cell line, demonstrate failure of inactivation similar to that seen in muscle from patients. Curiously, the mutated rat channel did not show the characteristic potassium sensitivity. This may have to do with the cell line used for expression; it may result from the absence of the β subunit; or it may be a difference between rat and human genes. Thus the genetic defects do produce the physiological defect.

Finally, Dr. Cannon mimicked the physiological defect with an anemone toxin that also produces inactivation failure. Rat muscle treated with toxin to produce moderate inactivation failure showed increased electrical excitability and slower relaxation of tension when stimulated, features characteristic of the myotonia that often precedes a paralytic attack in HPP.

The effects of more inactivation failure were studied with a computer model of excitation in muscle. This indicated that muscle can behave in three distinct manners, depending on the degree of inactivation failure: at low levels, excitation is normal and a patient would show no symptoms; at intermediate levels, hyperexcitability results, producing clinical myotonia; at high levels, the muscle is locked up at a higher voltage, permitting no excitation, and the patient is paralyzed. The physiological defect thus accounts for the disease.

Dr. Corey is also Associate Neurobiologist in the Department of Neurology at Massachusetts General Hospital, Boston, and Associate Professor of Neuroscience at Harvard Medical School.

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TRAFFIC OF SYNAPTIC VESICLES IN NEURONS AND ENDOCRINE CELLS

PIETRO DE CAMILLI, M.D., *Investigator*

Dr. De Camilli's research is aimed at elucidating mechanisms of membrane traffic in neurons and endocrine cells, with special emphasis on the traffic of synaptic vesicles (SVs). SVs are specialized organelles by which neurons secrete nonpeptide neurotransmitters at synapses. They are small secretory vesicles, highly homogeneous in size, that are clustered at nerve terminals in close proximity to release sites. They undergo exo-endocytotic recycling in nerve endings and, at each cycle, are reloaded with neurotransmitter content. The laboratory is focused on the elucidation of the biogenesis and exo-endocytotic recycling of SVs. (Some aspects of this work are carried out in collaboration with the laboratories of Dr. Reinhard Jahn [HHMI, Yale University] and Dr. Thomas Südhof [HHMI, University of Texas Southwestern Medical Center at Dallas].)

A central working hypothesis underlying Dr. De Camilli's work is that SVs are closely related to organelles found in endocrine cells and more distantly related to organelles found in all cells. The elucidation of molecular mechanisms involved in the life cycle of SVs may be of general relevance for the understanding of vesicular traffic in all eukaryotic cells.

A second focus of research in Dr. De Camilli's laboratory is the role of autoimmunity directed against SV proteins in human diseases.

Synaptic-like Microvesicles of Endocrine Cells

Recent work had shown that peptide-secreting endocrine cells contain a population of recycling microvesicles (synaptic-like microvesicles, SLMVs) that share important similarities with neuronal SVs. Many of the major membrane proteins of SVs are also present in the membrane of SLMVs. During the past year the properties of SLMVs were further investigated.

One line of research addressed the question of whether the recycling of membrane components of SLMVs involves the same endosomal intermediates that participate in the recycling of plasmalemma receptors (transferrin receptors). Results have suggested that membrane proteins of SVs and transferrin receptors transit through the same endosomes but are then sorted into distinct vesicular carriers. The relevance of these findings to the pathway of SV recycling in neurons is being investigated. In neurons, transferrin receptors are excluded from axons. Yet the mechanisms of endosomal sorting that operate in endocrine cells may also operate in nerve endings. (This work is supported in part by the National Institutes of Health.)

The possible function of SLMVs in neurotransmitter storage and uptake was studied, using pancreatic β cells as the model system. It was shown that SLMVs of these cells have a transporter for GABA (γ-aminobutyric acid) in their membranes. The pharmacological properties of this transporter are very similar to those of the transporter present in SVs of GABA-secreting neurons.

An Assay to Monitor Exo-endocytosis of SVs

An important step toward the elucidation of molecular mechanisms of SV exocytosis is the development of exocytotic assays independent of the measurement of neurotransmitter release. These assays will allow the monitoring of SV exocytosis in the absence of a synapse and irrespective of experimental manipulations that may affect the loading of SVs with neurotransmitters. An exocytotic assay based on antibodies directed against the luminal domain of the synaptic vesicle protein synaptotagmin was developed.

This assay has already permitted the determination that SVs undergo exo-endocytosis and recycling in the processes of developing hippocampal neurons in culture before the formation of synaptic contacts. The recycling that takes place in these immature neurons is very active, suggesting that the formation of a synapse correlates with a down-regulation of the rate of constitutive recycling. The regulation of SV exocytosis in developing neurons is being investigated.

Molecular Mechanisms of Exocytosis

The possibility of using yeast genetics to identify some of the proteins involved in SV exocytosis was explored. This experimental approach is based on the hypothesis that at least some basic feature of the exocytotic process may have been conserved in evolution, irrespective of whether the exocytotic event is regulated. A variety of temperature-sensitive yeast mutants defective in vesicle exocytosis (*sec* mutants), and therefore in growth, have been isolated in recent years. To identify proteins involved in the final states of the secretory pathway in mammalian cells, including neurons, Dr. De Camilli and his co-workers (in collaboration with the laboratory of Dr. Peter Novick, Yale University) searched mammalian gene products that rescue *sec* mutations. Rat brain

cDNAs were expressed in *sec4-8* yeast cells, which contain a temperature-sensitive allele of *SEC4*, and were screened for suppression of the growth defect at the restrictive temperature of 37°C. Sec4 is a member of the Sec4/Ypt1/rab branch of the Ras superfamily and is a protein involved in a late stage of the secretory pathway in yeast. A suppressing cDNA, *MSS4*, was isolated. *MSS4* encodes a 14-kDa protein that appears to enhance the function of *Sec4* by acting as a GDP-releasing protein. The Mss4 protein is similar in amino acid sequence to Dss4, a yeast protein with similar biochemical properties. (This research was supported in part by a grant from the National Institutes of Health.)

Autoimmunity to SV Proteins

The GABA-synthesizing enzyme glutamic acid decarboxylase (GAD) was found to be a major target of humoral autoimmunity in stiff-man syndrome (SMS) and in insulin-dependent diabetes mellitus (IDDM). SMS is a rare and severe central nervous system disease, while IDDM is a relatively common condition resulting from an autoimmune destruction of pancreatic β cells. GAD is represented by two similar proteins, GAD65 and GAD67. GAD65 is associated with the cytoplasmic surface of SVs of GABAergic neurons and of SLMVs of pancreatic β cells. During the past year Dr. De Camilli and his colleagues have established that GAD65 is the dominant autoantigen in SMS and have started to identify regions of the molecule that are the primary target of humoral autoimmunity.

Additionally, a brain protein, the target of humoral autoimmunity in three cases of SMS negative for GAD autoantibodies, was characterized. This protein is concentrated in nerve terminals, raising the possibility that SMS may be associated with autoimmunity directed against nerve terminal proteins rather than against a specific SV protein. (This work was supported in part by a grant from the National Institutes of Health.)

Dr. De Camilli is also Professor of Cell Biology at Yale University School of Medicine.

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MOLECULAR GENETICS OF THE STEROID RECEPTOR SUPERFAMILY

Ronald M. Evans, Ph.D., *Investigator*

It is axiomatic that the physiology of multicellular organisms depends on hormonal signals between cells that act through receptors to elicit changes in both cell shape and function. In the case of steroid and thyroid hormones and active metabolites of vitamins A (retinoic acid) and D, the receptors are intracellular transcription factors that activate or repress expression of target genes. Molecular studies by the Evans laboratory have revealed that these receptors are evolutionarily related and share an overall common structure that defines the steroid/thyroid hormone receptor superfamily. The laboratory has focused on the characterization of this hormone receptor family to provide insight into the origin and evolution of endocrine systems and to provide a simple means of characterizing the genetic networks that underlie cell fate and physiology.

Retinoid X Receptor

Derivatives of vitamin A, or retinoids, are a group of signaling molecules that are essential for several life processes, including growth, differentiation, and epithelial homeostasis. In the embryo, retinoic acid (RA) has been implicated in many roles, including the formation of the developing nervous system. RA exerts its effects on transcriptions for two classes of nuclear receptors, the retinoic acid receptors (RARs) and the retinoid X receptors (RXRs). The classification into the RAR and RXR subfamilies is mainly based on the differences in primary structure, sensitivity to synthetic retinoid ligands, and the ability to regulate expression of target genes.

A major question raised by the discovery of two retinoid-responsive systems is whether their functions are independent, interactive, or redundant. One approach to the answer is to determine whether these systems share common or discrete downstream target genes. In previous studies, the Evans laboratory has provided evidence that expression of the gene for the cellular retinol-binding protein type II (CRBPII) is dramatically induced by the RXR but not by the RAR. This discovery supported the suggestion that RXRs may be functionally distinct from the RAR system.

These data raise the question whether the two receptor systems recognize common ligands. This is particularly relevant because they are both activated by all-*trans* RA, but *in vitro*, only the RARs are capable of binding RA with high affinity. This discrepancy led the Evans group to propose that during a functional assay, cells convert all-*trans* RA to an RXR-specific ligand referred to as retinoid X. These observations also led them to develop a strategy to identify a metabolite of all-*trans* RA that might bind and activate RXR. It was not possible, in advance, to deduce how RA might be transformed, because the number of potential stereoisomers, conformers, and modifications could generate thousands of alternative molecules.

The Evans laboratory has recently described the identification of retinoid X as 9-*cis* RA, a naturally occurring RA stereoisomer. Although not previously seen in living organisms, 9-*cis* RA is evidently a prevalent vertebrate hormone. It represents the first such hormone identified in 20 years and the ninth hormone of the steroid/thyroid family of molecules.

RXR Heterodimers

Accessory factors present in nuclear extracts appear to be necessary for high-affinity binding of the RAR, thyroid hormone receptor (TR), and vitamin D receptor (VDR) to their cognate hormone response elements. It was proposed that these accessory factors might be members of the receptor family that stimulate receptor binding through heterodimer formation. Recently, in work partially funded by the National Institutes of Health, the Evans laboratory has found that RXR functions as a common heterodimeric partner for the VDR, TR, and RAR. These heterodimers display a high degree of cooperativity in binding to target DNA *in vitro*, as well as in augmenting functional activity *in vivo*.

Why the VDR, TR, and RAR interact with a common partner is not yet clear. Presumably, in this complex, RXR does not require its unique ligand (9-*cis* RA) but rather serves as a silent partner to promote hormone response of the associated receptor. It is clear that characterization of the RXR gene family, its patterns of expression, and the nature of the RXR ligand in controlling this process will further the understanding of the complex molecular nature of hormonal signaling.

Heterodimers in *Drosophila*

A *Drosophila* homologue of the RXR has been identified that corresponds to the *ultraspiracle* (*usp*) locus and is associated with an embryonic segmentation defect and larval lethality. The homology between *usp* and RXR is sufficient to suggest

that they might conserve potential heterodimeric functions. The Evans laboratory has recently demonstrated that *usp* can substitute for RXR in stimulating binding of the RAR, TR, and VDR to target DNA.

These observations led to the search for and ultimate identification of the ecdysone receptor (EcR) as a *Drosophila* partner of *usp*. Together, *usp* and EcR bind DNA in a highly cooperative fashion. Cotransfection of both EcR and *usp* expression vectors is required to render cultured mammalian cells ecdysone responsive. These results indicate that *usp* is an integral component of the functional EcR. By demonstrating that receptor heterodimer formation precedes the divergence of vertebrate and invertebrate lineages, these data underscore a central role for RXR and its homologue *usp* in the evolution and control of the nuclear receptor-based endocrine system.

Dr. Evans is also Professor at the Salk Institute for Biological Studies and Adjunct Professor of Biology and of Biomedical Sciences at the University of California, San Diego.

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GENETIC ANALYSIS OF PATHWAY AND TARGET RECOGNITION IN *DROSOPHILA*

COREY S. GOODMAN, PH.D., *Investigator*

The molecular mechanisms that control how neuronal growth cones find and recognize their correct targets during development continue to be the focus of Dr. Goodman's laboratory. Molecular genetic approaches in *Drosophila* are used to address these issues. Ongoing cellular analysis provides increasing knowledge about the pattern and identity of glia and neurons and the projections and pathways taken by neuronal growth cones. At the same time, monoclonal antibodies and other molecular probes have been generated that have revealed many of these cells and their processes (and have also been used to identify important genes). Based on this knowledge of the cell biology and availability of specific probes, members of the Goodman laboratory have been conducting a series of systematic mutant screens in order to identify genes that control growth cone guidance and target recognition in the *Drosophila* embryo.

Mutations That Affect Growth Cone Guidance in the Developing CNS

The first large-scale (near-saturation) screen for mutations that affect growth cone guidance was undertaken using a monoclonal antibody that reveals all central nervous system (CNS) axon pathways. Embryos from over 13,000 mutagenized and balanced lines were screened, and over 250 mutations were saved whose phenotypes suggest possible defects in growth cone guidance.

Mutations That Affect Guidance Toward or Away from the Midline

The growth cones of many CNS neurons initially head straight toward the midline, whereas other growth cones stay on their own side, leading to the hypothesis that midline cells play a key role in the formation of the axon commissures. The specificity and uniqueness of mutations in two genes, *commissureless* (*comm*) and *roundabout* (*robo*), make them excellent candidates to encode important components of mechanisms that guide growth cones toward or away from the midline. In both mutants the midline cells are present, and there is no indication of cell fate changes in the CNS. Rather, the primary defects appear to be in growth cone guidance.

Mutations in the *comm* gene lead to a CNS that lacks nearly all commissural pathways, even though longitudinal and peripheral pathways, sensory neurons, and muscles appear normal. In *comm* mutant embryos, the growth cones of commissural neurons do not project across the midline but instead extend only on their own side of the CNS.

Mutations in the *robo* gene lead to a specific and complementary misrouting, such that some growth cones that normally extend only on their own side of the CNS now project across the midline in one of the commissures. This mutant phenotype suggests that *robo* is part of a mechanism for keeping certain growth cones on their own side. There is considerable precedent in other organisms for repulsive signals for growth cone guidance. One model to explain these mutant phenotypes is that guidance at the midline relies on both attractive and repulsive cues. The *comm* gene product might be a component of an attractive signaling system; the *robo* gene product might be a component of a repulsive signaling system.

Mutations That Affect Guidance or Fasciculation in Longitudinal Pathways

Mutations have also been recovered that affect longitudinal axon pathways. Mutations in *longitudinals lacking* lead to embryos lacking most longitudinal axon pathways, although both commissural and peripheral pathways are present. The growth cones that normally pioneer longitudinal pathways initially extend but then stall, even though the longitudinal glia are present.

Other mutations display greater specificity for subsets of longitudinal pathways. For example, mutations have been recovered that affect the formation of the MP1 pathway, either causing the growth cone to stall or, in other mutations, to follow the wrong pathway.

Another gene, *fasciclin II* (*fas II*), plays a role in selective fasciculation of axons in the MP1 pathway. Initially identified from a monoclonal antibody screen, the fasciclin II protein is a member of the immunoglobulin superfamily and can function as a homophilic cell adhesion molecule. The protein is expressed on axons in the MP1 pathway and later on several other longitudinal axon pathways and on most motoneurons. In *fas II* mutant embryos, the pCC, vMP2, MP1, and dMP2 growth cones extend along the longitudinal glia but fail to fasciculate and do not form a tight bundle of axons.

The GAL4 enhancer trap method is being used to misexpress fasciclin II. When fasciclin II is ectopically misexpressed on sensory neurons in the periph-

eral nervous system, the aCC motoneuron can be misrouted, demonstrating that misexpression of fasciclin II can alter growth cone guidance.

A highly sensitive genetic assay has been established to screen for genes that encode products that interact with *fas II*. The assay utilizes a mutation that reduces the amount of fasciclin II protein to just the minimal amount required to generate certain sensory organs. This assay is used to screen for genes in which a 50% reduction in their protein level results in a failure of these sensory organs to form. One such gene is the *abelson* tyrosine kinase.

Mutations That Affect Motoneuron Guidance and Muscle Target Recognition

A model system for the study of both pathway and target recognition is based on the ability of motoneuron growth cones to find and recognize their correct muscles. Using an antibody that selectively stains the growth cones and axons of nearly all motoneurons, members of the Goodman laboratory have embarked on a systematic screen for mutations that affect motoneuron guidance and muscle target recognition. Thus far, over 3,000 mutagenized lines have been screened for the second chromosome. Many new mutants have been isolated that perturb the projection of motoneuron growth cones, including some that affect the development of specific motor axon pathways, some that perturb the formation of motor branches to specific muscles, and some that affect the recognition of specific muscle targets.

In other projects Dr. Goodman and his colleagues are studying the function of connectin during neuromuscular target recognition; of genes expressed by subsets of glia; of fasciclin IV during growth cone guidance in the grasshopper limb bud; of laminin A in the guidance of sensory axon projections and imaginal disc morphogenesis; and of the fat and dachsous cadherins in tissue morphogenesis and cell proliferation. (Studies on *comm*, *connectin*, and some of those on *fas II* are supported by grants from the National Institutes of Health.)

Dr. Goodman is also Professor of Neurobiology in the Department of Molecular and Cell Biology at the University of California, Berkeley, and Adjunct Professor in the Department of Physiology at the University of California School of Medicine, San Francisco.

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GENETIC CONTROL OF NEMATODE DEVELOPMENT

H. Robert Horvitz, Ph.D., *Investigator*

How do genes control animal development? Taking a primarily genetic approach to answer this question, members of Dr. Horvitz's laboratory have isolated developmental mutants of the nematode *Caenorhabditis elegans* and characterized them, using both genetic and molecular techniques. Because the complete ultrastructure, including the complete wiring diagram of the nervous system, and the complete cell lineage of *C. elegans* are known, mutant animals can be studied at the level of single cells and even single synapses. Genes involved in cell lineage, cell signaling, cell death, cell migration, and cell differentiation have been identified and analyzed.

Cell Lineage

The problem of cell lineage—how a single fertilized egg cell undergoes a complex pattern of cell divisions to generate a multiplicity of distinct cell types—is a major focus of this laboratory's research. Hundreds of cell lineage mutations have been identified that perturb the normal pattern of cell divisions and cell fates. Molecular studies of the genes defined by these cell lineage mutations suggest that much of the cell diversity that is generated in the course of the *C. elegans* development involves cascades of transcription factors.

For example, the genes *lin-26*, *lin-39*, and *lin-11* act sequentially during the cell lineages that generate the hermaphrodite vulva. The *lin-26* gene, which determines whether the Pn.p cells become neuronal or hypodermal, encodes a protein with two zinc fingers; *lin-39*, which determines whether hypodermal Pn.p cells become potential vulval cells or other hypodermal cell types, encodes a protein with a homeodomain; and *lin-11*, which determines which vulval cell fate a potential vulval cell will express, encodes another homeodomain-containing protein.

Cell Signaling

Much of *C. elegans* development involves cell signaling. The cell interactions that control the development of the hermaphrodite vulva have been analyzed in some detail. Studies in this laboratory have defined a genetic pathway for vulval development, which now consists of almost 50 genes. One of these genes, *let-60*, functions as a genetic switch during vulval induction. Dr. Paul Sternberg (HHMI, California Institute of Technology) and his colleagues showed that *let-60* encodes a Ras protein.

Based on this observation, Dr. Horvitz's laboratory discovered that mutations that activate *let-60* Ras in *C. elegans* are like those that cause oncogenic activation of Ras proteins in humans. The laboratory also identified a regulator of the *let-60* Ras gene, *sem-5*, and found that the gene encodes a novel signal transduction protein consisting entirely of SH2 and SH3 domains, protein regions originally observed in the mammalian *src* oncogene. The combined studies of Drs. Horvitz and Sternberg have revealed that the pathway of signal transduction in *C. elegans* vulval development is strikingly similar to that seen in mammalian cells and that many of the genes that function in cell signaling during vulval development correspond to mammalian oncogenes.

Cell Death

Naturally occurring or "programmed" cell death is common during the development of many animals, including *C. elegans*. Dr. Horvitz and his colleagues have identified and characterized genetically and molecularly two genes, *ced-3* and *ced-4*, that are necessary for the initiation of programmed cell death. Both of these genes act within the cells that die to cause their deaths. The gene *ced-9* negatively regulates the activities of *ced-3* and *ced-4* and protects cells against programmed cell death. Intriguingly, *ced-9* seems to be necessary for many, and perhaps all, cells that normally survive to avoid undergoing programmed cell death. The genes *ced-3*, *-4*, and *-9* have been cloned and are being analyzed molecularly. Three other genes, *ces-1*, *ces-2*, and *egl-1*, regulate *ced-9* in a cell-specific manner and thus determine whether specific cells live or undergo programmed cell death.

Seven additional genes, *ced-1*, *-2*, *-5*, *-6*, *-7*, *-8*, and *-10*, are necessary for the process of phagocytosis that causes the corpse of a cell undergoing programmed cell death to be engulfed by a neighboring cell. A number of other mutations have been identified that cause cells to die that normally survive, including some that result in a spongiform morphology reminiscent of certain human neurodegenerative diseases. Because cell death is the cause of the major clinical features of human neurodegenerative diseases, the genes responsible for familial amyotrophic lateral sclerosis (ALS; Lou Gehrig's disease) are being sought. Current evidence indicates that there are at least three such genes, one of which is located on chromosome 21.

Cell Migration

Animal development involves cell migrations. To understand what causes cells both to migrate and to stop migrating, this laboratory has analyzed a number of *C. elegans* cell migrations. One gene that controls a specific neuronal migration encodes a presumptive transcription factor of the zinc finger class, which suggests that this protein specifically regulates the expression of genes involved in this cell migration.

Five other genes control the migrations of the two sex myoblast (SM) cells, which are born in the posterior body region and move to a central position along the animal's length, near its gonad. These genes function within an intercellular signaling system that attracts the migrating SM cells to the gonad. At least one of these genes, *sem-5*, also acts in the signal transduction system that functions in the induction of the *C. elegans* vulva.

Cell Differentiation

Genes involved in the differentiation of both nerves and muscles have been identified. Some of these genes control the pioneering outgrowth of axons along the basement membrane. Other genes control neuron-neuron interactions along bundles of fasciculated neurons. One of these genes, *unc-76*, appears to encode a novel cytoplasmic protein. Other genes affect the acquisition of neuronal identity, the onset of neuronal differentiation, neurotransmitter expression, or synaptic function. (These studies were supported by a grant from the National Institutes of Health.)

Muscle differentiation has been examined by identifying mutants abnormal in muscle structure and function. Four genes appear to encode interacting components of a muscle membrane protein complex that regulates muscle contraction.

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MOLECULAR MECHANISMS IN THE REGULATION OF NEUROTRANSMITTER RECEPTOR FUNCTION

RICHARD L. HUGANIR, PH.D., *Associate Investigator*

Recent studies in many laboratories have provided evidence that protein phosphorylation is one of the major mechanisms in the control of synaptic transmission. Signal transduction at the postsynaptic membrane of chemical synapses is mediated by specific receptors that bind the neurotransmitter and transduce the signal to the interior of the cell. Dr. Huganir and his colleagues have been investigating the role of protein phosphorylation of neurotransmitter receptors in the regulation of synaptic transmission. They have used the nicotinic acetylcholine receptor (AChR), the prototypic neurotransmitter receptor, as a model system. In addition, they have been studying the role of protein phosphorylation in the regulation of the major excitatory and inhibitory neurotransmitter receptors in the brain, the receptors for glutamate and γ-aminobutyric acid.

Characterization of Protein Phosphorylation of the Nicotinic Acetylcholine Receptor

The AChR is a well-characterized neurotransmitter receptor that has served as a model system for the study of the structure, function, and regulation of neurotransmitter receptors and ion channels. The AChR is a pentameric complex of four types of subunits in the stoichiometry of $\alpha_2\beta\gamma\delta$. Studies in Dr. Huganir's laboratory have shown that the AChR is phosphorylated by cAMP-dependent protein kinase (PKA), protein kinase C (PKC), and a protein-tyrosine kinase. The phosphorylation of the receptor by cAMP-dependent protein kinase and the protein-tyrosine kinase has been demonstrated to regulate the rate of desensitization of the AChR. Desensitization, a common property of most receptors, is a process by which a receptor is inactivated in the presence of its neurotransmitter.

The functional effects of phosphorylation of the AChR are being studied in more detail by site-specific mutagenesis of the phosphorylation sites on the AChR subunits. All of the known phosphorylation sites have been mutated, and the mutant AChR has been expressed in *Xenopus* oocytes and human embryonic kidney (HEK) cells. Expression of the mutant subunits produces a fully assembled receptor, though the receptor is not phosphorylated. The mutant AChR has been shown to be functional using both intracellular and single-channel recording techniques. The desensitization kinetics of the mutant AChR are currently being analyzed and compared with the wild-type AChR.

Dr. Huganir's laboratory has investigated the neurotransmitters, hormones, and neuropeptides that regulate the level of AChR phosphorylation. A neuropeptide, calcitonin gene-related peptide (CGRP), which is a cotransmitter with acetylcholine at cholinergic synapses, has been found to regulate phosphorylation of the AChR by PKA. In addition, the neurotransmitter that regulates PKC phosphorylation of the AChR may be acetylcholine itself. Acetylcholine has been found to regulate AChR phosphorylation in an identical manner to that of phorbol esters, potent activators of PKC. The acetylcholine-induced phosphorylation is dependent on extracellular calcium, suggesting that calcium influx through the AChR is crucial for the activation of PKC.

The extracellular signals that regulate the tyrosine phosphorylation of the AChR have been elusive. However, studies in Dr. Huganir's laboratory funded by the National Institutes of Health have suggested that the presynaptic neuron is intimately involved in the activation of the protein-tyrosine kinase. In innervated rat diaphragm, the AChR is highly phosphorylated on tyrosine residues. However, denervation of the muscle leads to a progressive decrease in tyrosine phosphorylation. Moreover, during development, tyrosine phosphorylation of the AChR does not occur until after innervation of the muscle. Experiments using cocultures of chick myotubes and chick ciliary ganglion neurons have shown that innervation of the myotube *in vitro* stimulates tyrosine phosphorylation of the AChR. These results strongly suggested that something from the nerve, either a diffusible substance or the physical contact of the nerve terminal itself, activates tyrosine phosphorylation.

Recent studies in collaboration with Dr. Bruce Wallace (University of Colorado) have provided evidence that a neuronal extracellular matrix protein, agrin, may be the factor from neurons that regulates tyrosine phosphorylation of the AChR. Agrin, released by the nerve, mediates nerve-induced aggregation of the AChR at the synapse. Treatment of myotubes in culture with purified agrin stimulates tyrosine phosphorylation of the AChR and induces AChR aggregation. Moreover, inhibition of the agrin-induced tyrosine phosphorylation by tyrosine kinase inhibitors eliminates the agrin-induced aggregation of the receptor. These results suggest that tyrosine phosphorylation may mediate agrin-induced AChR aggregation and formation of the synapse.

To elucidate the mechanism of agrin's regulation of tyrosine phosphorylation of the AChR, Dr. Huganir and his colleagues have been studying the protein kinases and phosphatases that phosphorylate and dephosphorylate the AChR. To identify the protein-tyrosine kinase(s) that phosphorylates the receptor, they have used molecular cloning techniques. Polymerase chain reaction (PCR) techniques have been used to generate probes and isolate cDNA clones from *Torpedo* electroplax, a tissue highly enriched in the AChR. Several cDNAs for protein-tyrosine kinases were isolated, two of which are highly expressed in the electric organ. Using antibodies to these two abundant protein-tyrosine kinases, Dr. Huganir's laboratory was able to show that these kinases represented 60% of the organ's protein-tyrosine kinase activity. The two kinases are being characterized in greater detail to see whether they phosphorylate the AChR and whether their activity is regulated by agrin.

The level of tyrosine phosphorylation of the AChR may also be regulated by protein-tyrosine phosphatases that dephosphorylate the receptor. Using tyrosine phosphorylated AChR as a substrate, Dr. Huganir and his colleagues have identified the protein-tyrosine phosphatase in the *Torpedo* electric organ that dephosphorylates the AChR. They have recently purified this protein phosphatase 25,000-fold to homogeneity. This phosphatase is a 43-kDa protein that appears to be a novel enzyme, based on distinct chromatographic properties and its sensitivity to known inhibitors of other protein-tyrosine phosphatases. Using protein-sequencing techniques, Dr. Huganir and his colleagues have obtained the amino acid sequence for several peptide fragments of the phosphatase and are now isolating cDNAs that encode the phosphatase.

Regulation of Glutamate Receptors by Protein Phosphorylation

Dr. Huganir's laboratory has also been studying the regulation of the major excitatory receptors in the brain, the glutamate receptors. These receptors play a major role in synaptic plasticity, neuronal development, and neurological disorders. Glutamate receptors have been classified, according to their preferred agonists, into three groups: AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid), kainate, and NMDA (*N*-methyl-D-aspartate). Dr. Huganir's laboratory has been studying the role of protein phosphorylation in the regulation of the function of all three subtypes.

The regulation of glutamate receptors by protein phosphorylation has been examined using recombinant glutamate receptors expressed in mammalian cells and native glutamate receptors in neuronal cell cultures. Dr. Huganir's group has shown that PKA potentiates the kainate receptor subunit GluR6 by the direct phosphorylation of a single-serine residue in its major intracellular domain. In addition, they have found that the AMPA receptor subunit GluR1 is basally phosphorylated on serine residues by an unidentified protein kinase and is specifically phosphorylated on tyrosine residues when the receptor is cotransfected with pp60$^{v\text{-}src}$, a well-characterized protein-tyrosine kinase. Moreover, in recent studies the phosphorylation of native and recombinant NMDA receptors has been examined. The NMDAR1 subunit is directly phosphorylated and modulated by PKC.

Regulation of the GABA$_A$ Receptor by Protein Phosphorylation

Dr. Huganir's laboratory is also investigating the regulation of the major inhibitory neurotransmitter receptors in brain, the GABA$_A$ receptors. The phosphorylation of native and recombinant GABA$_A$ receptors has been studied. The α_1, β_1, and γ_2 subunits of the GABA$_A$ receptor have been expressed in HEK cells, and the effect of PKA and PKC phosphorylation has been studied.

Phosphorylation of the β_1 subunit on a single-serine residue by PKA decreases the peak GABA response and effects the desensitization kinetics of these recombinant receptors. In addition, PKC phosphorylates single-serine residues on the β_1 and γ_2 subunits of the GABA$_A$ receptor. Phosphorylation of these two serines also inhibits the GABA$_A$ receptor. Moreover, during the cloning of the murine GABA$_A$ receptor cDNAs, Dr. Huganir and his colleagues cloned a novel form of the γ_2 subunit (γ_2L) of the GABA$_A$ receptor. This subunit is generated by the insertion of eight amino acids in the major intracellular loop of the γ_2 subunit by differential splicing of the mRNA. This eight–amino acid insert encodes an additional PKC site.

Thus receptors consisting of the α_1, β_1 and γ_2L subunits contain three phosphorylation sites for PKC—one serine on the β_1 subunit and two serines on the γ_2L subunit. Phosphorylation of each of these sites is inhibitory, and with phosphorylation of all the sites, the peak GABA response of the receptor is barely detectable. These results demonstrate that the GABA$_A$ receptor is functionally modulated by PKC and that differential splicing of the γ_2 subunit regulates the sensitivity of the receptor to PKC modulation.

Summary

The results from Dr. Huganir's laboratory suggest that phosphorylation of neurotransmitter receptors

by various protein kinases serves to integrate the effects of convergent regulatory pathways on synaptic transmission. Moreover, these results suggest that protein phosphorylation is a major molecular mechanism for the regulation of neurotransmitter receptors and ion channel function and thus may play a primary role in the regulation of synaptic plasticity.

Dr. Huganir is also Associate Professor of Neuroscience and of Biological Chemistry at the Johns Hopkins University School of Medicine.

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MOLECULAR BASIS OF EXCITATION AND RECOVERY IN PHOTOTRANSDUCTION

James B. Hurley, Ph.D., *Associate Investigator*

Photoreceptor cells of both vertebrate and invertebrate retinas respond to light via highly sensitive G protein–mediated signal transduction cascades. Light hyperpolarizes vertebrate photoreceptors by stimulating hydrolysis of the intracellular second messenger cGMP. Light depolarizes invertebrate photoreceptors via production of the second messenger inositol trisphosphate. In both types of photoreceptor, the initial excitation phase of phototransduction is followed by recovery and de-

sensitization. Dr. Hurley and his colleagues are studying the molecular mechanisms of photoexcitation, recovery, and adaptation.

Vertebrate Phototransduction

Light initiates photoreceptor excitation by isomerizing the retinal chromophore of rhodopsin. Within the rod and cone cells of vertebrate retinas, photolyzed rhodopsin stimulates transducin, a photoreceptor-specific heterotrimeric G protein. Acti-

vated transducin α subunits bind GTP and stimulate a cGMP phosphodiesterase. The ensuing cGMP hydrolysis shuts down cGMP-gated cation channels within the photoreceptor outer-segment plasma membrane, blocking Na^+ and Ca^{2+} influx and hyperpolarizing the cell. Recovery occurs as transducin deactivates itself by hydrolyzing its bound GTP. Depletion of intracellular Ca^{2+} by a Na/Ca exchanger also contributes to recovery by stimulating a Ca^{2+}-sensitive guanylate cyclase to resynthesize cGMP.

Dr. Hurley and his colleagues are unraveling the molecular mechanisms of photoreceptor recovery and adaptation. In collaboration with Drs. Melvin Simon and Denis Baylor, transgenic mice that express a GTPase-deficient form of transducin α subunit in their rod photoreceptors were produced and analyzed. Mutant transducin accumulates in the outer segments of the transgenic rod cells at concentrations up to sixfold higher than endogenous transducin. To compensate for the presence of the mutant transducin, the rods respond by specifically and almost completely eliminating catalytic subunits of the transducin target, cGMP phosphodiesterase. Other phototransduction enzymes are unaffected, including rhodopsin, transducin β, phosphodiesterase γ, recoverin, arrestin, and phosducin. The altered rod cells show no signs of degenerating, but their electrical responses are slow and severely desensitized. These results show that the presence of persistently activated transducin stimulates a powerful adaptive feedback mechanism that specifically down-regulates its target enzyme.

During recovery cGMP is resynthesized by guanylate cyclase as the concentration of intracellular Ca^{2+} is lowered within the submicromolar range following photoexcitation. Dr. Hurley and his colleagues showed that recoverin, a 23-kDa Ca^{2+}-binding protein, stimulates a photoreceptor membrane guanylate cyclase only when the concentration of free Ca^{2+} is <200 nM. Recoverin was purified and examined for post-translational modifications by electrospray mass spectrometry. Its amino terminus was found to be heterogeneously acylated by one of four different types of short-chain fatty acid, C14:0, C14:1, C14:2, and C12:0. Similar heterogeneous acylation was also detected at the amino terminus of the transducin α subunit.

Dr. Hurley and his colleagues are investigating the functional role of heterogeneous amino-terminal acylation of recoverin and transducin. They found that acylated recoverin binds to phospholipid membranes in the presence of >1 μM Ca^{2+}, but nonacylated recombinant recoverin does not interact with membranes. The recoverin-membrane interaction relies on the binding of Ca^{2+}, which exposes the

acylated amino terminus of recoverin and allows the fatty acid to serve as a membrane anchor.

Recoverin activates membrane guanylate cyclase at Ca^{2+} concentrations <200 nM but dissociates from membranes under these same conditions. These two observations suggest that recoverin does not activate the cyclase directly. Dr. Hurley and his colleagues are investigating the possibility that additional factors participate in recoverin-mediated guanylate cyclase activation.

Drosophila Phototransduction

The mechanisms of phototransduction are quite different for invertebrates and vertebrates. In Drosophila photoreceptors, light stimulates a GTP-sensitive phospholipase C, which is essential for generating a photoresponse. Dr. Hurley and his colleagues have identified a unique Drosophila G protein β subunit, GBE, that may participate in Drosophila phototransduction. In situ hybridization and immunocytochemical analyses reveal that GBE mRNA and protein are photoreceptor specific.

The role of G protein β subunits in signal transduction is not well understood. In addition to presenting G protein α subunits to their receptors, β subunits may also regulate other effector enzymes. Dr. Hurley and his colleagues are investigating the role of GBE in Drosophila phototransduction. In collaboration with Dr. Charles Zuker (HHMI, University of California, San Diego), the laboratory has produced mutant Drosophila that are deficient in GBE. In situ biochemical analyses of these mutants reveal that GBE is essential for light-stimulated binding of GTP to a protein in Drosophila photoreceptors. Other enzymatic assays and electrophysiological analyses that may reveal the role of the G protein β subunit in Drosophila phototransduction are in progress.

Dr. Hurley is also Associate Professor of Biochemistry at the University of Washington School of Medicine, Seattle.

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MECHANISMS OF NEUROTRANSMITTER STORAGE AND RELEASE

REINHARD JAHN, PH.D., *Associate Investigator*

Dr. Jahn and his colleagues are interested in the mechanisms by which neurons store and release neurotransmitters. Neurotransmitters are stored in synaptic vesicles that are concentrated in nerve terminals and are released by Ca^{2+}-dependent exocytosis upon stimulation. Exocytosis occurs at specialized zones of the presynaptic plasma membrane (active zones). It is an extremely rapid event, indicating that all components required for membrane fusion are preassembled at the site of release. The mechanisms of membrane recycling are less well understood, but it is likely that it occurs via coated vesicles and early endosomes as intermediate stages.

During the past several years, Dr. Jahn's group has concentrated on a biochemical characterization of the membrane of synaptic vesicles. These studies provide the basis for a molecular characterization of individual steps of synaptic vesicle recycling, a line of research currently under development. The characterization of synaptic vesicle membrane components, carried out largely in collaboration with Dr. Thomas Südhof (HHMI, University of Texas Southwestern Medical Center at Dallas) and Dr. Pietro De Camilli (HHMI, Yale University) led to the identification of several unique protein families that are specifically localized on neuronal synaptic vesicles and related microvesicles in neuroendocrine cells. In addition, uptake and storage of amino acid neurotransmitters by synaptic vesicles was studied in some detail, focusing on the bioenergetic aspects of these processes.

Recently the work has concentrated on two of the synaptic vesicle proteins, synaptotagmin and rab3A. Synaptotagmin, which was cloned and sequenced in collaboration with Dr. Südhof, possesses two unique domains that are homologous to the regulatory C2 domain of protein kinase C. This domain is responsible for Ca^{2+} and phospholipid binding. A newly generated monoclonal antibody was used to affinity purify synaptotagmin to homogeneity and test its ability to interact with Ca^{2+} and phospholipids. The results show clearly that synaptotagmin is capable of high-affinity Ca^{2+} binding, provided that acidic phospholipids are present. The interaction apparently involves the formation of a ternary complex between synaptotagmin, Ca^{2+}, and phospholipid vesicles. This capacity is maintained when synaptotagmin itself is incorporated into liposomes. Under these conditions, Ca^{2+} at physiologically relevant concentrations induces vesicle aggregation but not fusion. Dr. Jahn and his colleagues assume that synaptotagmin is functioning as a Ca^{2+} receptor at the center of the fusion complex between synaptic vesicle membrane and plasma membrane. Membrane fusion itself presumedly involves additional proteins. The current efforts are concentrated on reconstituting exocytotic membrane fusion *in vitro*.

The rab3A protein is a representative of a subfamily of small GTP-binding proteins related to ras. It is specifically localized to synaptic vesicles. The rab proteins have been shown to be highly specific for intermediates in intracellular membrane traffic, where they are involved in the orderly and consecutive execution of individual trafficking steps. During exo-endocytosis, rab3A dissociates from the vesicle membrane and reassociates again at a later stage of the membrane cycle. Thus rab3A appears to be specific for vesicles ready to undergo exocytosis but lacking from endocytic vesicles. In agreement with this hypothesis, rab3A is absent from the membrane of coated vesicles. Recently the studies were extended to include the small GTP-binding proteins rab5 and rab7, which have been shown by others to be specific for early and late endosomes, respectively. Both these proteins are present in nerve terminals, where they are located on synaptic vesicle–derived membranes. However, the rab5- and rab7-containing membrane pool only partially overlaps with that containing rab3A. Thus these small GTP-binding proteins may serve as convenient tools

to dissect the synaptic vesicle cycle for functional studies.

Dr. Jahn is also Associate Professor of Pharmacology and Cell Biology at Yale University School of Medicine.

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MOLECULAR STUDIES OF VOLTAGE-SENSITIVE POTASSIUM CHANNELS

Lily Y. Jan, Ph.D., *Investigator*

Voltage-sensitive potassium channels represent a diverse group of ion channels that serve a variety of cellular functions, ranging from secretion control in certain animal cells to movement control of leaflets and stomal pores in plants. In the nervous system, potassium channels control excitability and modulate the strength of signaling between nerve cells; some potassium channels have been implicated in the processes of learning and memory. Since the cloning of *Shaker*, a potassium channel gene in *Drosophila*, a number of laboratories have cloned potassium channel genes from a variety of species ranging from snail to humans. The same basic design is present in all but one of these potassium channels, as well as in potassium channels in plants. Thus information concerning the structure-function relationship gleaned from studies of potassium channels such as those encoded by *Shaker* is likely to be of general interest. A summary of these structure-function studies is given below, followed by a progress report on the studies of potassium channel regulation in the mammalian brain.

Subunit Structure of a Potassium Channel

The polypeptides that form a potassium channel are much smaller than those that form a sodium or calcium channel and correspond to roughly one-quarter of the latter, suggesting that a potassium channel is likely to be a tetramer. Dr. Ehud Isacoff further showed that heteromultimeric potassium channels with novel properties form in *Xenopus* oocytes, if he injected into the oocytes either two different mRNA species or mRNA for a tandem dimer. Studies from several laboratories revealed that only polypeptides from the same potassium channel subfamily can form heteromultimeric channels; these polypeptides share ~70% amino acid identity in the hydrophobic core region, in contrast to the ~40% identity between polypeptides that belong to different subfamilies. Recently Dr. Min Li has shown that the hydrophilic amino-terminal domain of the *Shaker* potassium channel polypeptide is important in mediating the association of potassium channel subunits. Moreover, when he used the *Shaker* amino-terminal domain to replace the

amino-terminal domain of the mammalian DRK1 potassium channel polypeptide that belongs to a different subfamily, he detected formation of hetero-multimeric channels by this chimera and the *Shaker* potassium channel polypeptide. Thus the hydrophobic core region of the DRK1 and *Shaker* polypeptide are compatible in the subunit interactions, even though they only share 40% amino acid identity; the failure of the wild-type *Shaker* and DRK1 polypeptides to coassemble and form functional channels can be attributed to incompatible interaction between their hydrophilic amino-terminal domains.

Structural Elements Involved in Specific Potassium Channel Functions

Voltage-sensitive potassium channels display a number of intriguing properties. They contain intrinsic voltage sensors that can detect the electrical potential difference across the membrane. In response to appropriate potential changes, these sensors presumably will cause conformation changes that open the channel. The duration that a channel stays in the open state is controlled by a process called inactivation. Previous studies have revealed the presence of a cytoplasmic inactivation gate (ball-and-chain), which is thought to interact with a receptor at the cytoplasmic mouth of the channel pore after the channel opens, thereby blocking ion permeation and causing channel inactivation. While a potassium channel is open, potassium ions go through the channel pore $\sim 1,000$ times more readily than do other physiologically relevant ions. This high level of selectivity has to be achieved in a way that is compatible with the large ionic flux: >1 million ions can go through a channel in a second. Structure-function studies carried out in several laboratories including that of Dr. Jan have begun to associate individual structural elements with specific channel functions.

The hydrophobic core region of a potassium channel polypeptide contains seven segments of predominantly hydrophobic residues: S1 through S6 and the H5 sequence between S5 and S6. The latter has been suggested to form part of the channel pore, based on studies by other laboratories. The S4 sequence contains basic residues at every third or fourth position and is present in voltage-gated sodium, calcium, and potassium channels. The proposal that the S4 sequence functions as a voltage sensor of the channel is consistent with the observation that mutagenesis of either basic or hydrophobic residues of the S4 sequence in the *Shaker* channel specifically affects the voltage-dependent proper-

ties of channel gating, as shown by Drs. Diane Papazian, Leslie Timpe, and George Lopez.

The cytoplasmic inactivation gate of the *Shaker* potassium channel has been associated with the amino terminus of the *Shaker* polypeptide; Dr. Richard Aldrich (HHMI, Stanford University) has shown that, while deletions of residues from the amino terminus reduce or eliminate fast inactivation, cytoplasmic application of a peptide of the sequence of the *Shaker* amino terminus restores inactivation. Similar observations have been made by Dr. Tim Baldwin and Dr. Lopez for rat *Shal1* (Kv4.2), a mammalian A-type potassium channel gene belonging to a subfamily different from that of *Shaker*.

The receptor for the inactivation gate is likely to include five residues in the S4-S5 loop that are highly conserved among all four subfamilies of voltage-gated potassium channels, as shown by studies by Dr. Isacoff. Mutations of these residues either increase or decrease the affinity between the inactivation gate and its receptor. These mutations also reduce both the inward and the outward potassium ion flux through a single channel pore. Recent studies by Dr. Paul Slesinger further reveal that the selectivity between different permeant ions may be altered by mutations in the S4-S5 loop. These observations strongly suggest that the S4-S5 loop is at or near the cytoplasmic opening of the potassium channel pore. (The structure-function studies of the *Shaker* potassium channel are supported by the National Institutes of Health.)

Potassium Channels in the Mammalian Brain

A surprisingly large number of potassium channel genes have been found to be expressed in the mammalian brain; at least 15 such genes have been reported that give rise to voltage-gated potassium channels of fairly similar properties in the *Xenopus* oocyte expression system. Drs. Meei-Ling Tsaur and Morgan Sheng have characterized the expression patterns of several potassium channel genes in the rat brain. Overlapping but clearly distinct patterns of gene expression have been found. Moveover the expression patterns of some potassium channel genes are dynamic in the adult brain; the mRNA levels of Kv1.2 and Kv4.2 show a transient decrease in the excitatory granule cells of the dentate gyrus hours after pentylenetetrazole-induced neuronal activities. Thus individual neurons may express different subsets of potassium channel genes and thereby acquire their particular characteristics of excitability.

Recent studies by Drs. Sheng and Baldwin have further revealed that certain potassium channel proteins are differentially localized to either dendrites

and cell bodies or axons and possibly terminals. Thus different potassium channels of similar biophysical properties may be targeted to different subcellular compartments of a neuron and thereby become specialized with respect to their functional role. (The studies of potassium channels in the mammalian brain are supported by the National Institute of Mental Health.)

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NEURAL DEVELOPMENT IN *DROSOPHILA*

YUH NUNG JAN, PH.D., *Investigator*

Dr. Jan and his colleagues are interested in the mechanisms of cell determination and differentiation during neural development. How do neurons arise from undifferentiated ectodermal cells? What gives the neurons their individual identities in terms of shape and function? How are neuronal pathways initially established? The long-term goal is to understand these processes at the molecular level. A genetic approach is being used; i.e., the studies begin with the isolation of *Drosophila* mutants that affect neurogenesis, neuronal type, or axonal pathway formation. Identification of the mutations can lead to the isolation of genes important in neural development.

Studies by this group and others have identified a number of genes that specify cell fate in the embryonic sensory nervous system of *Drosophila*. The genes appear to act at different steps progressively to restrict the cells to more and more determined fates, as outlined below.

Early during embryogenesis, at the cellular blastoderm stage, a group of genes called proneural genes determine where the nervous system develops. Proneural genes are expressed in clusters of cells and thereby endow those cells with the potential to form neuronal precursors. As a neuronal precursor forms, it inhibits neighboring cells from doing so. This lateral inhibition involves the action of neurogenic genes. The commitment of neuronal

precursors may involve the actions of certain neuronal precursor genes, which lock a cell into a particular fate. The identity of a neuronal precursor is further specified by the neuronal-type selector gene.

Proneural Genes

The proneural genes define a state that makes cells competent to become neuronal precursors. The prototype of proneural genes are the genes of the *achaete-scute* complex (*AS-C*). Recent studies from the groups of Drs. Juan Modolell, Alain Ghysen, and Sean Carroll (HHMI, University of Wisconsin) provide strong support for the notion of proneural genes.

Previous studies indicate that there should be other proneural genes in addition to *AS-C*. The *Drosophila* sensory nervous system consists of three major types of sensory organs: 1) the es (external sensory) organ, 2) the ch (chordotonal) organ, and 3) the md (multiple dendrite) neuron. All the es organs and the majority of md neurons require *AS-C* for formation, whereas ch organ formation is independent of *AS-C*. Presumably there is an additional proneuronal gene required for ch organ formation. Drs. Yves Grau and Andy Jarman found this missing proneural gene, *atonal*, based on the fact that all known proneural genes contain a basic helix-loop-helix (bHLH) motif. They lined up all the known bHLH sequences, designed primers for polymerase chain reactions, and managed to clone *atonal*. *atonal* has the predicted properties: 1) its expression pattern prefigures the location of ch organs, 2) deletion of the chromosomal region containing *atonal* results in a failure of ch organ formation, and 3) ectopic expression of *atonal* leads to formation of ectopic ch organs.

Neurogenic Genes

Previous studies from the laboratory of Dr. José Campos-Ortega have shown that removing the function of any of the six known zygotic neurogenic genes—*Notch* (*N*), *Delta* (*Dl*), the *Enhancer of split* complex [*E(spl)C*], *mastermind* (*mam*), *neuralized* (*neu*), and *big brain* (*bib*)—leads to hypertrophy of both the central and peripheral nervous systems, presumably as a result of losing lateral inhibition. These six neurogenic genes can be placed into two separate genetic pathways. *N* and *Dl* belong to one pathway. Work from the laboratories of Drs. Spyridon Artavanis-Tsakonis (HHMI, Yale University) and Michael Young (HHMI, Rockefeller University) strongly suggests that the protein products of *N* and *Dl* mediate a receptor-ligand interaction between neighboring cells. *bib* also encodes a membrane protein, like *N* and *Dl*, but belongs to a separate genetic pathway. Phenotype analysis revealed that *bib* mutants are qualitatively different from *N* and *Dl* null mutants. In *N* and *Dl* mutants, all cells in the proneural clusters develop into neuronal precursors, whereas in *bib* mutants only a subset of cells do so. This suggests that cells in proneural clusters are not completely equivalent. The sequence of *bib* shows considerable similarity with certain transporter molecules, suggesting that *bib* may also function in controlling such transport.

Neurogenic genes were named as such because they were originally identified by their function in neural development. However, recent studies revealed that they have a much broader function. Dr. Young's laboratory found that neurogenic genes are involved in specifying muscle cell fate. Dr. Hannele Ruohola-Baker in Dr. Jan's laboratory found that neurogenic genes also control follicle cell fates during oogenesis. Thus neurogenic genes as a group are involved in selecting a subset of cells among a group of equivalent, pluripotent cells to take on a specific fate. These neurogenic genes may be considered as a functional cassette that mediates cell interaction in various developmental contexts, including neurogenesis, oogenesis, and muscle development.

Neuronal Precursor Genes

Once a cell is singled out to become a neuronal precursor, it develops differently from its neighbors. Dr. Jan's laboratory identified several neuronal precursor genes (including *prospero* and *deadpan*) that are expressed in neuronal precursors but not their surrounding ectodermal cells. They proposed that neuronal precursor genes have important roles in directing neuronal precursor differentiation; each gene may control a subset of neuronal properties. Consistent with this hypothesis, it has been found that *prospero* appears to control aspects of axonal outgrowth.

Analysis of the neuronal precursor gene *deadpan* provided an unexpected dividend. It is known from the work of Dr. Calvin Bridges that sex in *Drosophila* is determined by the X chromosome to autosome ratio (X/A ratio). Previous studies by Dr. Tom Cline demonstrated that *sex lethal* (*Sxl*) is the master regulatory gene of *Drosophila* sex determination. In male (X/A = 0.5) *Sxl* is off, and in female (X/A = 1) *Sxl* is on. There are numerator genes that count the X chromosome. It was not known whether a denominator gene(s) that counts the autosomes also exists. Susan Younger-Shepherd in Dr. Jan's laboratory found that *deadpan* is in fact a denominator gene. An interesting link between *Drosophila* neural de-

velopment and sex determinations has become apparent. The two developmental pathways share a number of key regulatory genes: *scute*, *daughterless*, *deadpan*, and *extramacrochaete*. This provides yet another example that a set of genes can be considered as a functional cassette, which carries out analogous function in different developmental contexts.

Neuronal-type Selector Gene(s)

Neuronal-type selector genes control the type of sensory neuron to which a precursor will give rise. For example, the *cut* locus is required for es organs to acquire their correct identity. In the absence of *cut* function, the es organs are transformed into ch organs. Recent experiments by Dr. Karen Blochlinger suggest that *cut* functions as a binary switch. If *cut* is misexpressed in cells that are normally ch precursors, they will develop into es organ-like structures. This suggests that expression of the *cut* gene activity determines whether the precursor will develop into an es or ch organ. The *cut* product contains a homeodomain and is likely to act as a transcription factor regulating the expression of downstream differentiation genes.

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CONTROL OF CELL PATTERN IN VERTEBRATE NEURAL DEVELOPMENT

Thomas M. Jessell, Ph.D., *Investigator*

Research in Dr. Jessell's laboratory continues to focus on two aspects of neural development in vertebrates: defining the interactions that control cell identity and regional pattern during the initial stages of neural development, and characterizing the molecules and mechanisms that guide developing axons.

Control of Neural Cell Identity and Pattern

The development of the nervous system begins with the induction of the neural plate from uncommitted ectodermal cells in response to signals from mesoderm. As the neural plate folds to form the neural tube, distinct classes of cells appear in a bilaterally symmetric manner at different dorsal-ventral (D-V) positions. This pattern is conserved at different segmental levels of the spinal cord, suggesting that cell identity in this region is defined primarily by position along the D-V axis of the neural tube.

Studies by Dr. Toshiya Yamada, in collaboration with Drs. Marysia Placzek and Jane Dodd, have suggested that the bilateral organization and D-V pattern of cell types is established by signals derived from cells at the midline of the neural plate. *In vivo* grafting studies and *in vitro* assays that monitor functional and biochemical markers of the floor plate have shown that the differentiation of neuroepithelial cells into floor plate is induced by contact with the notochord. Strikingly, the newly induced floor plate acquires the inductive properties of the notochord, including the ability to induce floor plate differentiation. This self-inductive signal may be important in recruiting cells into the floor plate from more lateral regions of the neural plate.

The floor plate and notochord appear to control the position and identity of motor neurons and other ventral spinal cord neurons. Grafts of notochord or floor plate next to the neural tube induce the appearance of ectopic motor neurons and other ventral neurons, defined by cell-specific markers. Inversely, removal of the notochord and floor plate prevents the differentiation of these neuronal types. In contrast to the contact dependence of floor plate induction, the differentiation of motor neurons *in vitro* can be initiated by diffusible signals from the floor plate and notochord. These findings suggest that D-V patterning in the spinal cord is dependent on a cascade of inductive interactions, initiated by the notochord and continued by the floor plate.

Genes that control cell identity in the neural tube. Studies to identify genes that might contribute to the determination of cell identity in the neural tube have focused on two classes of molecules: transcription factors and peptide growth factors.

Dr. Ariel Ruiz i Altaba has shown that cells at the midline of the neural plate that later become the floor plate express DNA-binding proteins related to the rat HNF-3 and the *Drosophila fork head* gene families. Overexpression of one of these genes, *Pintallavis*, in *Xenopus* embryos leads to the expression of a floor plate–specific gene, F-spondin, in dorsal regions of the spinal cord and to the loss of dorsal cell types. These results suggest that *Pintallavis* contributes to the specification of floor plate properties.

Studies by Dr. Yamada, in collaboration with the laboratory of Dr. Thomas Edlund, have shown that developing motor neurons express the *Islet-1* gene, a member of the LIM-homeodomain family. *Islet-1* expression occurs soon after the final division of motor neurons but before the onset of expression of other neuron markers, suggesting that the gene may control some of the later phenotypic properties of motor neurons.

There is increasing evidence that secreted growth factors of the transforming growth factor-β (TGF-β), Wnt, and fibroblast growth factor (FGF) families may control neural cell identity and pattern. Dr. Konrad Basler and Dr. Yamada have identified a novel member of the TGF-β superfamily, Dorsalin, which is selectively expressed in the dorsal region of the neural tube. Grafting experiments show that signals from the notochord and floor plate appear to restrict Dorsalin expression to the dorsal spinal cord. The localized expression of the Dorsalin gene may be required for the differentiation of cell types found in the dorsal region of the neural tube.

In addition, repression of Dorsalin expression in ventral regions may be a prerequisite for the differentiation of ventral cell types such as motor neurons. In support of this idea, Dorsalin prevents the differentiation of motor neurons *in vitro* in response to notochord and floor plate–derived signals. Dorsalin may block motor neuron differentiation by causing ventral neural tube cells to acquire dorsal cell fates or may arrest neuroepithelial cell differentiation.

Regulation of Axonal Pathfinding in the Central Nervous System

The projection of axons to their targets along complex but stereotyped pathways is initiated soon

after the commitment of cells to a particular neuronal fate. Studies in Dr. Jessell's laboratory have focused on the mechanisms that control the growth and guidance of axons of commissural neurons in the developing spinal cord. The complex trajectory of commissural axons appears to reflect the operation of several distinct cues that guide axons over discrete segments of their overall path.

Commissural axons express the cell surface glycoprotein TAG-1. Molecular cloning of TAG-1 revealed that it is a member of the immunoglobulin superfamily closely related to L1 and NCAM (neural cell adhesion molecule). In contrast to L1 and most forms of NCAM, TAG-1 is attached to the axonal surface by a lipid anchor and is also released from cells in large amounts, suggesting that the protein is present in the environment of extending commissural axons. Studies by Drs. Andrew Furley, Mary Hynes, and Dan Felsenfeld have found that TAG-1 can promote cell aggregation by a homophilic interaction but promotes neurite outgrowth by a heterophilic interaction, possibly with members of the integrin family. Since TAG-1 is likely to be released from pioneering commissural axons, it may provide a local substrate that both promotes and restricts spatially the growth of later differentiating commissural axons. The guidance of pioneering commissural axons appears, however, to be dependent on cues other than TAG-1, including a floor plate–derived chemoattractant.

When commissural axons reach the contralateral boundary of the rat floor plate, they turn orthogonally and grow parallel to the longitudinal edge of the floor plate in a rostral direction. This change in trajectory is accompanied by the loss of TAG-1 from the axonal surface, the onset of expression of high levels of L1, and a marked increase in axon fasciculation. These changes occur in contact with the floor plate, raising the possibility that cell surface or extracellular matrix molecules synthesized by the floor plate might mediate these events, either through adhesive interactions or intercellular signaling.

Studies by Drs. Avihu Klar and Jochen Walter have begun to characterize adhesion or signaling molecules expressed selectively by the floor plate. Molecular cloning has identified two novel proteins that are expressed at high levels by the floor plate over the period of commissural axon growth. The first, F-spondin, is a secreted protein that exhibits homology to the extracellular matrix glycoprotein thrombospondin and to other adhesive proteins. The second, FP-84, is a transmembrane protein that is a novel member of the immunoglobulin family. Both F-spondin and FP-84 have neurite outgrowth-promoting activity. The restricted expression of these two proteins on the surface of the floor plate or in the extracellular matrix may contribute to the changes in guidance and behavior of commissural axons at the ventral midline of the spinal cord.

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CELL AND MOLECULAR MECHANISMS OF LEARNING

Eric R. Kandel, M.D., *Senior Investigator*

Molecular Mechanisms of Memory in *Aplysia*

Dynamics of cAMP and protein kinase A (PKA) subunits in Aplysia *sensory neurons.* Binyamin Hochner, Bong-Kiun Kaang, and Eric Kandel, in collaboration with Brian Bacskai, Martyn Mahaut-Smith, Stephen Adams, and Roger Tsien (HHMI, University of California, San Diego), injected cyclic AMP–dependent protein kinase, labeled with fluorescein and rhodamine on the catalytic and regulatory subunits, respectively, into *Aplysia* sensory neurons either in culture or in intact cell clusters. Confocal fluorescence microscopy revealed that bath application of serotonin (5-HT) produced striking gradients of cAMP—high in the processes, low in the central bodies of the neurons. Perinuclear increases in cAMP slowly caused translocation of the freed catalytic subunits into the nucleus to an extent linearly related to the percentage dissociation of the kinase. The diffusional processes may act as a filter to ensure that only repeated or particularly strong synaptic stimulation of the cAMP cascade moves sufficient catalytic subunits into the nucleus to phosphorylate transcription factors and activate gene expression.

Transcriptional activation. One hallmark of most types of long-term memory is the requirement for new protein synthesis. In long-term sensitization of the gill-withdrawal reflex in *Aplysia*, this requirement can be studied on the cellular level in the monosynaptic connections between the sensory and motor neurons of this reflex. Here, long-term but not short-term facilitation requires new protein synthesis and is reflected in an altered level of expression of specific proteins regulated through the cAMP second messenger pathway.

Based on gene transfer into individual sensory neurons of *Aplysia* in the intact nervous system, Kaang, Kandel, and Seth Grant found that 5-HT induces transcriptional activation of a reporter gene driven by the cAMP response element (CRE), that this induction requires CRE-binding proteins (CREBs), and that it is blocked by competing CRE oligonucleotides. The induction by 5-HT does not occur following a single pulse of 5-HT but becomes progressively more effective following two or more pulses. Moreover, expression of GAL4-CREB fusion genes shows that 5-HT induction requires phosphorylation of CREB on Ser[119] by PKA but not by the calcium/calmodulin-dependent kinase. These data provide supporting evidence for CREB-modulated transcriptional activation with long-term facilitation.

Molecular Mechanisms of Long-Term Potentiation

Genetic analysis reveals that the fyn *tyrosine kinase gene is necessary for LTP and learning in mice.* Tyrosine kinase inhibitors block long-term potentiation (LTP) in the CA1 region of the hippocampus. To identify specific tyrosine kinases involved in LTP, Grant, Thomas O'Dell, Kevin Karl, Paul Stein, Phillipe Soriano (HHMI, Baylor College of Medicine), and Kandel screened mice with mutations engineered in either of four cytoplasmic tyrosine kinase genes: *fyn, src, yes,* and *abl*. Although these four kinases are coexpressed in the hippocampus, only the *fyn* mutant mice failed to show normal LTP. With low-intensity tetanic stimulation, slices from *fyn* mutant mice showed essentially no LTP. However, a modest amount of LTP could be induced using higher-intensity (75% of the maximum excitatory postsynaptic potential [EPSP]) tetanic stimulation ($133.2 \pm 9.3\%$ of control), but this potentiation was smaller than that observed in slices from control animals ($168.5 \pm 11.6\%$ of control). By contrast, synaptic transmission appeared normal in slices from *fyn* mutant mice, and paired-pulse facilitation was not different from that observed in control animals.

The impairment of LTP appears to correlate with impaired spatial learning in the Morris water maze, suggesting a functional link between LTP and spatial memory. In addition to its importance in LTP, the *fyn* gene is also necessary for the normal development of the pyramidal cell layer of the hippocampus, since the layer in the CA3 region shows structural abnormalities. Together these data suggest that the *fyn* tyrosine kinase is important for the induction of LTP and implicate a new biochemical pathway contributing to synaptic plasticity.

Nitric oxide produces long-term enhancement of synaptic transmission in the CA1 region of the hippocampus by an activity-dependent mechanism. There is evidence that the membrane-permeant molecule nitric oxide may act as a retrograde message during LTP in the hippocampus. A difficulty with the retrograde message idea, however, has been that lateral spread of a diffusible message could lead to potentiation of transmission at inactive presynaptic terminals, which would violate the observed pathway specificity of LTP. A possible solution to this problem would be for the effects of the message to be restricted to recently active presynaptic fibers. Scott Small, Kandel, and Robert

Hawkins tested this possibility by applying nitric oxide to hippocampal slices either alone or coincident with weak presynaptic stimulation (50 Hz for 0.5 s).

Application of 100 nM nitric oxide alone, presynaptic stimulation alone, or nitric oxide 5 min after presynaptic stimulation ("unpaired" training) produced no significant long-term effects on the field EPSP recorded in the CA1 region. However, when nitric oxide was applied at the same time as the presynaptic stimulation ("paired" training), the synaptic potential was immediately enhanced and remained enhanced for at least an hour. Paired training still produced significant long-term enhancement of the EPSP in the presence of APV, a specific blocker of postsynaptic N-methyl-D-aspartate (NMDA) receptors, which blocks the induction of LTP by tetanic stimulation.

These results are consistent with the hypothesis that nitric oxide acts as a retrograde message with activity-dependent presynaptic effects during LTP. This mechanism would be formally similar to activity-dependent presynaptic facilitation in Aplysia and activity-dependent neuromodulation more generally.

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A MULTIFACETED G PROTEIN–COUPLED RECEPTOR BIOASSAY

Michael R. Lerner, M.D., Ph.D., *Associate Investigator*

The superfamily of seven-transmembrane domain receptors linked to G proteins provides a major biological mechanism for detecting intercellular and environmental signals. While the range of ligands for these receptors is vast and examples include photons, odorants, monoamines, peptides, and prostaglandins, the receptors themselves utilize a variety of intracellular signaling systems, such as the activation of adenyl cyclase or phospholipase C or the inhibition of adenyl cyclase. Because the receptors are often situated at control points for crucial activities, understanding the molecular basis of how li-

gands interact with them is important for the development of therapeutically useful chemicals. To address this problem, Dr. Lerner and his colleagues have developed a multifaceted, G protein–coupled receptor bioassay.

The G protein–coupled receptor bioassay is based on the ability that many animals have to change their color rapidly. In nature, color changes are used for purposes such as camouflage and to communicate states of emotion. A major way in which these changes are effected is by the controlled movement of pigment granules within chromatophores. When pigment granules in melanophores (a particular type of chromatophore) are aggregated, the animal appears light. When pigment is dispersed, the animal appears dark. The pigment translocation apparatus is controlled via second messenger systems that are regulated by G proteins. As a result, the state of pigment disposition within melanophores reflects the state of activity of G protein–coupled receptors.

This laboratory has created an immortalized frog melanophore cell line that forms the center of an assay for monitoring the activity of G protein–coupled receptors. To test the system, β_2-adrenergic, substance P, and dopamine 2 receptors were initially expressed in the melanophores. Normally, β_2 receptors elevate intracellular cAMP, substance P receptors raise inositol 1,4,5-trisphosphate (IP_3) and calcium, and dopamine 2 receptors lower cAMP. Stimulation of either the β_2 or substance P receptors induces pigment dispersion, while stimulation of the dopamine receptor leads to pigment aggregation. Eight exogenous receptors have been expressed in the pigment cells, and all actuate appropriate pigment translocation following stimulation. Using 96 well plates containing cells expressing a receptor of interest, it is possible to obtain detailed dose-response curves and EC_{50} and IC_{50} values for many drugs.

Future studies that will involve investigating the effects of site-specific mutations on receptor function will be aided by the ability to analyze the functional activation of G protein–coupled receptors in large numbers of cells while retaining single-cell resolution. For this purpose, digital image processing has been integrated into the melanophore-based bioassay. Translocation of melanosomes within thousands of individual pigment cells can be simultaneously tracked by capturing gray scale video images before and after receptor activation. Digital

subtraction of poststimulation from prestimulation images generates bitplane images containing pixels with nonzero gray scale values wherever melanosome movement occurs. Up to 200,000 plasmids can be evaluated in <1 h for the presence of ones coding for functional receptors.

To facilitate investigations of chemicals for their properties as ligands for G protein–coupled receptors, a means of creating a mobile peptide library (MPL) containing large numbers of soluble peptides that are individually localized has been developed. The value of such libraries stems from the observation that the natural ligands for many G protein–coupled receptors are peptides. If large numbers of distinct peptides can be compared for their abilities to activate or block specific receptors, much could be learned. The starting point for constructing an MPL is a synthetic peptide combinatorial library (SPCL) where peptides are tethered to resin beads. However, for a functional assay, it is necessary for ligands to be capable of freely interacting with receptors residing on living cells. To create an MPL, the bonds anchoring synthetic peptides to the beads on which they were constructed, as well as those between the amino acid side chain protecting groups and the peptides, are severed in a dry state. Because the peptides remain attached, albeit noncovalently, to their source beads, they can be manipulated as discrete units for a variety of functional tests.

The combination of the melanophore-based G protein–coupled receptor bioassay and the availability of MPLs will be useful for unraveling how ligands stimulate or block seven-transmembrane domain receptors.

Dr. Lerner is also Associate Professor in the Departments of Internal Medicine (Section of Rheumatology) and of Pharmacology and the Child Study Center at Yale University School of Medicine.

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BASIC MECHANISMS OF VOLTAGE-DEPENDENT ION CHANNELS

CHRISTOPHER MILLER, PH.D., *Investigator*

Ion channels are the most basic elements of molecular hardware in the nervous system. They are the membrane-spanning proteins that directly mediate the transmembrane ionic fluxes giving rise to electrical signals in neurons and other electrically active cells. All proteins of this type have the same structural plan: that of a water-filled pore spanning the cell membrane. Thus channels act as leakage pathways for ions down their thermodynamic transmembrane gradients. The ion channels involved in neuronal function select strongly among the different species of inorganic ions in the aqueous solutions bathing the cell membrane. In addition, channels must have the ability to open and close their conduction pores in response to external signals, such as binding of neurotransmitters (ligand-gated channels) or changes in transmembrane electric field (voltage-dependent channels).

Dr. Miller's research is aimed at questions of fundamental molecular mechanisms of ion channel operation and of the underlying protein structures. The absence of direct structural information makes it necessary to draw inferences from close examination of function. This can be done because ion channels, unique among proteins, can be studied at the single-molecule level, both in the cellular environment with patch-recording techniques and after reconstitution into biochemically defined "artificial" membranes. Dr. Miller's laboratory is currently focusing on several voltage-dependent ion channels that provide opportunities to address mechanistically important questions about channel structure and function.

Peptide Neurotoxins as Probes of K$^+$ Channel Structure

Charybdotoxin (CTX), a peptide derived from scorpion venom, blocks a small family of K$^+$-specific channels. Having recently shown that CTX acts as a physical plug in the channel's outer "mouth," Dr. Miller is currently using the peptide as a probe of this important region of K$^+$ channels. Work on the *Drosophila* Shaker K$^+$ channel, using site-directed mutagenesis, has identified residues that specifically and locally alter the binding of CTX. These residues are therefore located near the ion entryway, a region of the protein contributing to the transmembrane pore.

The toxin binds to the high-conductance Ca^{2+}-activated K$^+$ channel with high affinity, and the interaction of CTX with this channel may be studied at high resolution by reconstituting single channels into planar bilayer membranes. Recently, in a research project supported by the National Institutes of Health, Dr. Miller and Dr. Per Stampe have expressed a gene coding for CTX at high levels in *Escherichia coli*. The recombinant peptide is rendered fully active by several *in vitro* post-translational modifications. Specific residues required for channel blocking have been identified by mutagenesis of the synthetic gene followed by single-channel analysis.

These studies are enhanced by knowledge of the solution structure of CTX, recently determined by two-dimensional nuclear magnetic resonance (NMR). With this approach, the entire surface of the toxin has been functionally mapped. The functionally important residues all lie on the same side of the molecule and thus define an interaction surface that is recognized by the receptor in the channel mouth.

Dr. Miller and Dr. Steven Goldstein are currently seeking to use CTX as a molecular caliper to determine physical distances between residues in the mouth of a voltage-gated K$^+$ channel from *Drosophila*, the genetically manipulable Shaker. While the naturally occurring Shaker channel is only weakly sensitive to CTX, a mutated channel has been engineered to build a highly sensitive CTX receptor into the channel's outer "vestibule." Now, with the functionally critical residues on both toxin and channel known, the aim is to pair these up, to identify specific residues forming interaction partners in the toxin-channel complex. This is now being approached by complementary mutagenesis of both channel and toxin. With the toxin structure known, identification of only a few such residues will place strong constraints on the channel's structure.

An additional use of CTX as a structural probe of a K$^+$ channel is being carried out by Dr. Miller and Amir Naini, a graduate student. Previous results identify Lys27 as a mechanistically rich residue, in that the ϵ-amino group protrudes slightly, from the channel-bound toxin into the narrower K$^+$ conduction pathway. This ϵ-amino group can sense the presence of K$^+$ ions within the selectivity regions of the pore. Naini has now shown that Lys27 may be replaced by a chemically reactive Cys, and that unnatural lysine analogues may be replaced at this position while maintaining toxin activity. Thus "tethered" with different chain lengths, amino groups on toxin

analogues at position 27 will serve as sensitive, well-calibrated probes of ionic interactions inside the channel pore.

Purification and Reconstitution of Voltage-Dependent Cl⁻ Channels

The electric ray *Torpedo californica* carries in its electric organ a Cl^--specific channel with an unusual structural characteristic: the channel is built as a dimeric, or double-barreled complex, with two identical Cl^- diffusion pathways in a single molecular unit. Dr. Miller and Dr. Richard Middleton, in a project partially supported by the National Institutes of Health, are using a functional assay for this channel protein in a solubilized state for protein-level purification and active-site labeling studies.

At this point the protein has been purified at analytical scale, using a monospecific immunoaffinity column, and the subunit structure of the complex is being investigated. The longer-range goal is to scale up the purification to exploit the huge advantage of the electroplax: the high level at which the protein is expressed in this organ. In principle, it should be possible to use this source for routine purification of tens of milligrams of voltage-dependent Cl^- channel protein.

High-Level Expression of Ion Channel Proteins

Over the past few years, ion channel genes have been cloned at a rapidly accelerating pace, and they can be expressed in systems that allow high-sensitivity electrophysiological assays. But it would be desirable to have a high-level expression system available for producing milligram-scale protein for cloned channel genes. This is a fundamental stumbling block for future molecular attacks on ion channels at the level of protein biochemistry. For this reason, Dr. Miller is using the Shaker K^+ channel as a test for high-level expression in several heterologous systems, including baculovirus-infected insect cells, mammalian cells, yeast, and transgenic mice (and eventually goats) with the channel targeted to the mammary epithelium. The rationale here is that although this line of work is highly empirical, the channel under test, which contains an engineered high-affinity CTX-binding site, is well suited for the goals. Specifically, it lends itself to straightforward immunoaffinity purification, and the toxin-binding site allows a clear distinction between total expressed protein and assembled tetrameric channel.

Ion Conduction in Ca²⁺-activated K⁺ Channels

Dr. Miller, with Qiang Lu, a graduate student, has recently begun to apply single-channel reconstitution methods, developed earlier, to a genetically manipulable Ca^{2+}-activated K^+ channel, the "slow-poke" channel of *Drosophila*. This project is now in its infancy, but the directions are clear: to identify, through mutagenesis and single-channel analysis, the tight binding sites for K^+ and Ba^{2+} ions residing within the conduction pore. These experiments will initially require the development of routine techniques to transfer expressed channels to the planar lipid bilayer system, where the necessary Ba^{2+}-K^+ interactions can be measured.

Dr. Miller is also Professor of Biochemistry at Brandeis University, Waltham, and Adjunct Professor of Molecular Biology at Massachusetts General Hospital, Boston.

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CORTICAL PROCESSING OF VISUAL MOTION INFORMATION

J. Anthony Movshon, Ph.D., *Investigator*

The primate visual cortex consists of more than two dozen separate areas, many of which are known to be specialized for particular forms of visual analysis. One of the best-understood segments of this pathway contains a series of connected areas thought to be involved in visual motion processing. The lowest levels in this subpathway contain neurons that make local measurements of image speed and direction; higher levels of the pathway combine these local signals to perform more complex analyses and to make motion signals available to the motor system for the control of behavior.

Dr. Movshon's laboratory has been active for a number of years in the study of neuronal signals in this part of the visual cortex, concentrating on a particular visual area called MT or V5, which occupies a pivotal position in the processing sequence. Recently, Dr. Movshon and his colleagues have concentrated on two main questions: How are the signals that are carried by MT neurons used to support perceptual judgments of motion? How are such signals used to generate visuomotor behavior?

MT Signals and Visual Motion Perception

In collaboration with Dr. William T. Newsome's laboratory (Stanford University), Dr. Movshon has used statistical techniques based on the theory of signal detection to analyze the information carried by individual MT neurons, recorded while trained monkeys performed a psychophysical task tailored to make maximum use of the signals carried by the neuron under study. This combined behavioral and electrophysiological technique makes it possible to measure neuronal and psychophysical performance in parallel under strictly controlled conditions.

Most recent models of visual cortex have stressed the distributed nature of neuronal processing, leading to the expectation that the signals of individual neurons might not be particularly informative. These experiments revealed, however, that most MT neurons, in fact, carry enough information to support the psychophysical performance actually observed in trained monkeys and humans. Although this result does not rule out the idea that combinations of signals across large numbers of neurons may be useful for other tasks, it suggests that for appropriately constructed circumstances, perceptual judgments may be based on signals carried by a handful of neurons.

MT Signals and Visuomotor Behavior

In addition to their role in supporting the perception of motion, signals carried by MT neurons have rather direct access to the portions of the motor system involved in the generation and control of smooth pursuit eye movements. These movements are used by primates to stabilize the image of a moving target on the retina so that it may be properly seen. The signals that drive pursuit have long been known to carry information about the speed and direction of target motion. Recent studies of pursuit have provided much information about the dynamic properties of the visual signals needed to support these precise eye movements; and in collaboration with Dr. Stephen G. Lisberger (University of California, San Francisco), Dr. Movshon has been studying the visual response properties of MT cells to establish whether their signals carry the requisite information.

These experiments have particularly explored the dimension of stimulus acceleration, since theoretical analysis has shown that target acceleration signals are likely to be needed to generate fast and accurate pursuit. The results have revealed that the dynamics of MT responses are well suited to the task of providing inputs to the motor system and that at least half of the neurons also carry the necessary signals about target acceleration. This outcome strengthens the idea that MT signals are used rather directly to support pursuit.

A Fly in the Ointment

Before concluding that pursuit is inextricably linked to signals carried by MT neurons, it is necessary to take account of another set of results from Dr. Movshon's laboratory. In these experiments (in collaboration with Dr. Lisberger and with Dr. Michael J. Hawken at New York University), pursuit eye movements were measured in human observers viewing specially constructed targets designed to isolate particular components of the visual pathway. One particular target was created by modulating the color but not the luminance of the display. The visual motion pathway in general, and MT cells in particular, are known to be relatively insensitive to such isoluminant color targets, leading to the expectation that these targets would elicit inferior pursuit. In fact, isoluminant targets are precisely as effective as conventional luminance targets in driving pursuit. This

suggests that while MT signals provide a part of the foundation for this class of visuomotor behavior, other pathways as yet undescribed physiologically must also contribute.

Dr. Movshon is also Professor of Neural Science and Psychology at New York University and Adjunct Professor of Physiology and Biophysics at New York University School of Medicine.

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SIGNAL TRANSDUCTION AND PROCESSING IN THE OLFACTORY SYSTEM

RANDALL R. REED, PH.D., *Associate Investigator*

The mammalian olfactory system is an exquisitely sensitive sensory organ responsible for encoding information on the intensity and the identity of chemical stimuli. The initial events in olfactory signal transduction occur in a complex sensory organ, the nose. Molecules that comprise the chemical stimuli perceived as odors are first solubilized and concentrated by protein components of the aqueous medium that bathes the tissue. The neuroepithelium that lines the nasal cavity contains the sensory neurons responsible for the conversion of the external stimulus into an electrical signal. Each of these sensory neurons extends a dendritic process to the luminal surface, where a small number of cilia extend into the mucous layer. These cilia, the presumed site of odorant recognition, likely contain the machinery required for signal transduction. Considerable electrophysiological evidence has accumulated to suggest that individual receptor neurons respond differently to each odor. The axons extend from the cell bodies of the sensory neurons located in the epithelium and project on particular second-order neurons in the glomerular tufts of the olfactory bulb.

One of the most remarkable aspects of the olfactory neurons is their ability to be replaced from a population of precursor cells. This replacement of olfactory neurons from neuroblast precursors occurs continually in adult animals. More significantly, acute injury to the olfactory bulb or to the receptor neurons leads to the rapid loss of the sensory cells and their subsequent, synchronous replacement. Complex regulatory mechanisms must underlie this neuronal replacement and the formation of appropriate connection between the epithelium and the olfactory bulb.

The Mechanism of Olfactory Signal Transduction: A G Protein–coupled Cascade

Receptor proteins present in the cilia membranes of the sensory neuron are presumed to provide the specificity of odorant recognition. These receptor proteins might then converge on a common intracellular pathway. The membrane-bound receptors that couple to G proteins in a wide variety of systems share considerable structural similarities. Each member of the family is glycosylated and crosses the membrane seven times. Recently, Drs. Linda Buck and Richard Axel (HHMI, Columbia University) have identified several new members of a large G protein–coupled receptor family expressed exclusively in olfactory epithelium that likely encode the odorant receptor proteins. Currently, Dr. Reed's laboratory is examining the genomic structure of this family to understand the mechanism by which expression of individual receptors is directed to particular cells. Preliminary results indicate that an individual receptor is expressed in perhaps only 0.1% of the sensory neurons. Expression of these receptor proteins in heterologous systems may help to elucidate the relationship between the structure of the receptors and the odorant ligands that they bind.

Dr. Reed has identified several other components in the presumptive pathway for olfaction. The labo-

ratory has characterized a GTP-binding protein, G_{olf}, exclusively expressed in the olfactory neurons and localized to the sensory cilia. This olfactory-specific G protein shares some homology to transducin, the G protein involved in visual signal transduction. The cyc⁻ variant of the S49 mouse lymphoma cell line is deficient in GTP-stimulated adenylyl cyclase activity and has proved to be a useful system to investigate G protein function. When G_{olf} is introduced into this cell line, GTP-dependent adenylyl cyclase activity is restored. Moreover, the ability of a β-adrenergic agonist, isoproterenol, to stimulate adenylyl cyclase is also restored. These data suggest that G_{olf} can couple heterologous receptors to adenylyl cyclase.

The third component in the signal transduction cascade, adenylyl cyclase, is expected to be abundant in olfactory cDNA libraries. At the level of enzyme activity, there are 10-fold higher levels of this protein in olfactory tissue homogenates than in brain tissue. In a collaboration with Dr. Alfred Gilman, Dr. Reed's laboratory has identified cDNA clones encoding three distinct forms of adenylyl cyclase. One form is expressed exclusively in brain, and a second is expressed in several peripheral tissues. A third form of the enzyme is expressed only in olfactory epithelium. This protein, type III adenylyl cyclase, is largely confined to the cilia of rat olfactory neurons. Biochemical experiments performed by expressing type III adenylyl cyclase in a mammalian expression system revealed that the enzyme has unusually low basal activity. This would be advantageous for a protein that is highly concentrated in the cilia and yet must maintain low resting levels of intracellular second messenger.

Regulation of Olfactory-Specific Gene Expression

The expression of components of the olfactory signal transduction pathway and other proteins specifically expressed in the sensory neurons is likely to be regulated coordinately. Dr. Reed's laboratory has initiated a series of experiments to identify the cis-acting regulatory sequences that control expression of this set of genes. An 11-base sequence present in each of these genes appears to bind a protein present only in extracts from olfactory tissue. By using a yeast expression/selection scheme, a candidate protein for a trans-acting transcription factor operating at this site has been identified. Experiments are now under way to establish a functional role for this protein in olfactory neuronal differentiation.

Genetic Approaches to the Functional Analysis of Signal Transduction Components

Elucidation of a function for the variety of forms of adenylyl cyclase expressed in mammalian tissues may prove difficult. In research supported by the National Institute of Mental Health, Dr. Reed's laboratory has initiated experiments in *Drosophila melanogaster* to identify homologues of the mammalian proteins. Among the loci that appear to encode adenylyl cyclase in *Drosophila* is the learning and memory mutant *rutabaga*. This gene in *Drosophila* has been cloned and the mutation in memory-deficient flies identified. Biochemical studies have revealed that a single base change in the coding region for the *rutabaga* protein leads to a complete loss of enzyme activity. Demonstration that this defect is the cause of the defect in learning and memory was achieved by analyzing insertions of a transposable element into the same gene. The patterns of gene expression in the fly and the genetic consequences of these mutations confirm that the cloned *Drosophila* cyclase is *rutabaga*. The study of the molecular defects at this locus may expand understanding of the mechanism of memory processes.

The identification and molecular characterization of components of the cAMP second messenger pathway provide important new tools to examine the relationship between structure and function of these proteins. Additionally, the availability of olfactory neuron–specific isozymes of many of the proteins in the cascade will allow the development of useful systems for the reconstitution of the odorant detection pathway in cell lines amenable to the study of the mechanisms of specificity and sensitivity.

Dr. Reed is also Professor in the Departments of Molecular Biology and Genetics and of Neuroscience at the Johns Hopkins University School of Medicine.

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EXTRACELLULAR FACTORS AFFECTING NEURON DEVELOPMENT

Louis F. Reichardt, Ph.D., *Investigator*

Dr. Reichardt's laboratory studies soluble and adhesion-promoting factors that regulate survival and differentiation of neurons during development and regeneration of the nervous system.

Development of Axons in the Primary Visual Projection

In past work, laboratory members examined adhesive interactions promoting axon growth by retinal neurons over astroglial substrates *in vitro*, as a model to discover interactions likely to be important *in vivo* for normal development of the retina and the retinal tectal projection. These experiments suggested that several adhesive receptors on the growth cones of the axons of retinal ganglion cells promote axon growth. These receptors include three cell adhesion molecules—NCAM, N-cadherin, and L1/NgCAM—and several integrin heterodimers.

During the past year, Dr. David Sretavan has extended these studies to the endogenous pathway along the neuroepithelium traversed by retinal ganglion cell axons. To label these axons, the embryonic eye has been injected with lipophilic fluorescent dyes that are incorporated into retinal ganglion cell membranes. With these dyes, fluorescence imaging has been used to monitor movements of individual growth cones in the optic stalk, the portion of the visual pathway between the retina and optic chiasm. The early growth cones in this pathway exhibit regular movements toward the future optic chiasm. Individual growth cones move over the neu-

roepithelial substrate and at early times are not extensively fasciculated with each other. Injection of antibodies to L1 inhibits their movements, indicating that at least one of the adhesion-promoting molecules important *in vitro* is also important *in vivo*.

The behavior of these growth cones has also been examined in the optic chiasm, a major choice point in the visual projection. From there each growth cone projects either contralaterally or ipsilaterally, depending primarily on the position of its cell body in the retina. In the developing optic chiasm, growth cones contact each other intermittently, but primarily grow in contact with the neuroepithelial cell substrate. Numerous examples of contra- and ipsilaterally projecting growth cones have been visualized. When ipsilaterally projecting growth cones turn, they reorient within 10–20 minutes. These decisions always appear to occur in the chiasm, and observation suggests that they depend on local information present in the chiasm.

Dr. Sretavan has identified a population of early-developing neurons that are candidates to regulate the growth of retinal ganglion cell growth cones in the chiasm and in more-distal regions of the visual projection. These neurons are localized in the vicinity of the future optic chiasm and project to the dorsal surface of the embryonic midbrain, the approximate region innervated by retinal ganglion cells, at times in development preceding the arrival of retinal ganglion cell growth cones at the optic chiasm. In double-labeling experiments, growth cones from

retinal ganglion cells appear to contact the somata and axons of these chiasmic neurons. Thus these neurons are strong candidates to provide growth-promoting and directional cues for retinal ganglion cell growth cones in the optic chiasm and in the optic tract pathway.

In collaboration with Dr. Ellen Pure of the Rockefeller University, Dr. Reichardt and his colleagues have shown that the neurons in the optic chiasm express a hyaluronic acid–binding receptor named H-CAM or CD44. This has made it possible to remove these neurons by injection of CD44-specific antibodies and complement. In the absence of these neurons, growth cones of retinal ganglion cells fail to enter the optic chiasm and can be visualized as long as four days later at the junction between the optic stalk and chiasm. In collaboration with Mark Siegel of Genentech, it has been possible to show that the morphology of the neuroepithelium is normal after ablation of the chiasmic neurons. Thus the results argue that these neurons are necessary for normal development of the visual pathway. Although there is no evidence at present supporting a direct role for CD44 in visual pathway development, the possibility is being actively investigated.

Work has continued on characterizing receptors for extracellular matrix (ECM) proteins in the retina and visual projection. Dr. Blaise Bossy previously isolated cDNAs encoding a novel integrin α subunit named α_8 that associates with the β_1 subunit and is strongly expressed on axons in the primary visual projection and many other axon tracts. He has expressed the $\alpha_8\beta_1$ receptor but has not yet identified an ECM protein as a ligand. Michael DeFreitas has obtained strong evidence that the integrin $\alpha_3\beta_1$ functions as a receptor for thrombospondin. Both $\alpha_3\beta_1$ and thrombospondin II are expressed in the embryonic retina. Dr. Barbara Varnum-Finney has evidence that a β_1 integrin also functions as a receptor for tenascin, another ECM protein that is strongly expressed in the embryonic central nervous system.

Regulation of Integrin Receptor Function in Neurons

The functions of some integrins are down-regulated on neurons at times correlating with target innervation and are up-regulated by manipulations that prevent or disrupt target innervation. Dr. Ivan de Curtis has found evidence in the neuroretina for both transcriptional and post-translational regulation of the functions of laminin-binding integrins. In the past year, function-blocking antibodies and cytochemistry have been used to extend this work.

Two major laminin receptors—$\alpha_6\beta_1$ and $\alpha_3\beta_1$—are expressed in developing retina. Antibody inhibition experiments show that the $\alpha_6\beta_1$ receptor accounts for most but not all interactions of early retinal neurons with laminin. At later developmental stages, this receptor is expressed on neurons that bind laminin poorly. Binding to laminin is enhanced by the antibody TASC, which activates integrins by binding to the β_1 subunit. Binding to laminin is abolished by inhibitory antibodies to β_1 and is reduced by inhibitory antibodies to α_6.

The results imply that integrins can exist in more than one activity state on neuronal cell surfaces. In the early developing retina, $\alpha_6\beta_1$ is present on neurons and neuroepithelial cells in a comparatively active conformation. At later times the same integrin is present in a comparatively inactive state. Thus integrin activity is developmentally regulated. Transcription of α_6 is also regulated during development and also contributes to changes in neuronal responsiveness to laminin. In collaboration with this laboratory, Dr. Clayton Buck (University of Pennsylvania) has shown that the TASC monoclonal antibody binds an epitope in the "neck" of the integrin spatially distant from the ligand-binding pocket. Work in progress seeks to understand the mechanisms by which the this antibody activates the functions of these integrins. Since regulators of activity can in principle cause rapid changes in integrin function that quickly affect growth cone guidance, the laboratory is also seeking to identify endogenous mediators of integrin activity.

Integrins in *Caenorhabditis elegans*

In collaboration with Dr. Cynthia Kenyon's laboratory (University of California, San Francisco), Sonya Gettner has identified an integrin β subunit that is widely expressed in *C. elegans*. The sequence, genomic organization, and genomic position of this protein's gene are now known. The protein associates with at least three putative integrin α subunits. Cytochemistry indicates it is widely expressed throughout the *C. elegans* life cycle.

In collaboration with Dr. Ed Hedgecock (Johns Hopkins University), it has been possible to show that a gene, *pat-3*, encodes this integrin β subunit. Ms. Gettner has sequenced several mutants in this gene and has shown that each introduces a nonsense or missense mutation into the β subunit. The mutations have distinct phenotypes but affect cell attachment, cell morphogenesis, cell migration, and process outgrowth by many different cell types. The mutations include a null, a partial deficiency, and a probable neomorph. Thus this system may be valu-

able for studying integrin functions in a genetically accessible organism.

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MOLECULAR MECHANISMS OF DEVELOPMENTAL AND REGULATED EXPRESSION OF NEUROENDOCRINE GENES

Michael G. Rosenfeld, M.D., *Investigator*

Molecular Mechanisms of Anterior Pituitary Gland Development

Defining the mechanisms by which specialized cells arise and generate organs containing functionally diverse cell types is a fundamentally important issue in understanding development. Therefore a major research focus in the laboratory has been to define the molecular mechanisms that dictate the developmental and regulated expression of neuroendocrine genes and to begin to apply these principles to analysis of neuronal gene expression. Dr. Rosenfeld and his colleagues have initially utilized the anterior pituitary as a model for understanding the molecular mechanisms involved in generating specific cell phenotypes within an organ.

Rathke's pouch, the pituitary precursor, becomes committed to specific organ development several days before the expression of markers of individual cell types within the mature gland. Between the time of organ commitment and organ maturation, a series of cell-type-specific differentiation and proliferation events occur, generating five cell types in the mature anterior pituitary gland defined by the trophic factors that they synthesize and secrete. The anterior pituitary arises from ectodermal cells adjacent to the anterior neuropore that involute to make contact with the neuroectoderm that gives rise to the hypothalamus. This contact is the only region of mesodermal incompetence between ectoderm and neuroectoderm in the primitive head, enabling the resultant cell-cell contact to serve as the critical in-

ductive event. Coincident with these cell-cell contacts on embryonic day 11 (E11) in the rat, Dr. Rosenfeld and his colleagues found that the first known anterior pituitary marker, the α-glycoprotein subunit transcript, is restricted to most or all cells in a single layer of epithelium in a clear posterior-anterior gradient, suggesting an asymmetry of the inducing factor(s).

A fundamental issue is whether cells that might fail to express the α-glycoprotein subunit marker at this stage can serve as precursors of specific cell types. Because the five cell types subsequently arise in a precise temporal and spatial pattern, the signals that trigger the appearance of each cell type from the apparently homogeneous primordium remain a question. While individual cell types arise in a stratified fashion in the developing gland, the five mature cell types are more homogeneously distributed throughout the anterior pituitary. This could reflect either a loss of homophilic interactions between cell types or the action of migration-inducing factors. The initial appearance of differentiated cell types within restricted portions of the anterior pituitary may indicate that specific signals induce progenitor cells to differentiate or proliferate agents from other tissues or the pituitary itself. Corticotrophs and thyrotrophs arise on E14, with gonadotrophs arising on E17. Although the structurally related prolactin and growth hormone genes are ultimately expressed in discrete cell types (lactotrophs and somatotrophs, respectively), their initial

expression occurs on E17–E18 in the central caudal stem cells.

High levels of cell-specific expression of the rat prolactin gene are dictated by two separate regions —a distal enhancer (−1830 to −1530) and a proximal region; mutation of even a single cis-active element in either gene can reduce gene expression by 90–98%. Structurally similar elements within the initial 180 bp of the growth hormone promoter that act in a position- and orientation-dependent fashion are required for cell-specific expression of heterologous genes in transgenic mice. The consensus binding site for this critical cis-active element bound a tissue-specific POU domain transcription factor, activating both a 60–amino acid divergent homeodomain and a 76– to 78–amino acid region of homology with several other transcriptional or developmental factors (Oct-1 and Oct-2, and unc-86) that may specify cell lineage relationships. Dr. Rosenfeld and his colleagues have shown that the POU-specific domain of Pit-1 activates DNA, is required for high-affinity site-specific binding, and is important in cooperative, DNA-dependent dimer binding on native sites. Consistent with Pit-1 regulating the prolactin and growth hormone gene activation, the onset of expression of Pit-1 protein on E15.5 correlates closely with that of the prolactin and growth hormone genes. However, the failure of the dwarf animals to develop thyrotrophs, which appear more than a day before detectable Pit-1 gene expression, suggests that Pit-1 may be involved in thyrotroph survival or maintenance, and indeed thyrotrophs appear normally on E13 in Snell dwarfs.

Mouse genetics has provided direct evidence for the developmental role of the tissue-specific transcription factors. A variety of developmental mutants have been identified by their dwarf phenotypes. Among these are the Snell, Jackson, and Ames dwarf mutants that exhibit a virtually identical phenotype: they contain no lactotrophs, somatotrophs, or thyrotrophs and have markedly hypoplastic anterior pituitary glands. Jackson and Snell dwarfs are allelic on chromosome 16, and it has been demonstrated that both dwarf phenotypes result from mutations of the Pit-1 gene. The Jackson mutation is a rearrangement, whereas the Snell phenotype is a transversion mutation in the Pit-1 POU homeodomain that alters a crucial residue in the DNA recognition helix, conserved among all homeodomain proteins. The mutation impairs the ability of Pit-1 to bind to its DNA recognition elements. The expression of thyrotroph embryonic factor (TEF), a basic-leucine repeat transcription factor, and not that of Pit-1, correlates spatially and temporally with the onset of thyroid-stimulating hormone-β (TSHβ) ex-

pression in the rostral tip and can activate the TSHβ promoter expression in heterologous cell types. These data indicate that Pit-1 is necessary for the specification of phenotype of three cell types within the anterior pituitary and directly link a transcription factor to commitment and progression events in mammalian organogenesis. The hypoplastic nature of the dwarf pituitary indicates that cell proliferation is a critical component of the developmental program specified by Pit-1. However, a complex combinatorial code is responsible for the initial activation of the Pit-1 gene. In a collaborative project with Dr. John Parks, a point mutation (a transfer) that alters a single residue in the POU-specific domain of human Pit-1 results in short stature with absent growth hormone and prolactin gene expression but a normal-sized gland. This implies distinct requirements for activation of distal target genes and the Pit-1–dependent genes that are required for cell proliferation.

Pit-1 positively autoregulates the expression of its promoter as a consequence of binding to two Pit-1 elements. These data are consistent with a positive, attenuated autoregulatory loop that appears to function as a molecular memory in maintaining Pit-1 gene expression.

Combinatorial positive and negative events ultimately lead to cell-specific expression of the growth hormone and prolactin genes in distinct cell types. Based on a synergistic interaction with Pit-1 activating the prolactin gene distal enhancer, the estrogen receptor appears to be another critical regulator.

Pit-1 actions are modulated in the mature pituitary gland. Pit-1 is a phosphoprotein regulated by cAMP and phorbol esters, with phosphorylation at two specific residues (Ser^{115}, Thr^{220}). One functional consequence of this post-translational modification is to alter affinity for cognate Pit-1 DNA recognition element. The direction of the altered affinity is site dependent, with marked inhibition of binding on growth hormone sites but actually increased binding to the Pit-1 autoregulatory site.

A Large Family of POU Domain Proteins in Mammalian Brain Development

Dr. Rosenfeld and his colleagues subsequently cloned multiple additional mammalian members of the POU domain gene family and demonstrated that all of the known POU domain genes are expressed during neural development and exhibit precisely restricted temporal and spatial patterns of gene expression. In this regard, the POU domain family of transcription factors resembles the developmental patterns of the hierarchy of regulatory genes that are sequentially activated during *Drosophila* develop-

ment. Analysis of cortical and sensory system development suggests potential roles for these factors in determining mature neuronal phenotypes. With support from the National Institute of Mental Health, Dr. Rosenfeld and his colleagues have identified seven additional, novel POU domain factors, most of which are expressed during neural development. However, several recently identified genes are expressed only in distinct organs outside of the central nervous system. Analysis of the binding sites for these novel POU domain factors reveals that they bind primarily as monomers to sites distinct from those of Pit-1. A detailed analysis revealed that a spacing rule dictated binding of these proteins to these cognate DNA sites. Thus the POU-specific domain and POU homeodomain are bipartite DNA-binding domains, with each making sequence-specific major groove contacts. The spacing between these, based on minor groove contacts, is distinct for each class of POU domain factors. This spacing rule could provide a critical determinant of the specificity of the target genes for each class of POU domain protein. Aspects of these projects were also supported by grants from the National Institutes of Health.

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DETERMINATION OF NEURONAL CELL FATES IN THE DEVELOPING FLY EYE

GERALD M. RUBIN, PH.D., *Investigator*

Dr. Rubin's laboratory studies various aspects of gene expression and differentiation in *Drosophila*, with emphasis on molecular and genetic approaches to neurobiology. The current work focuses on the development of the visual system, and in particular, on understanding the signal transduction pathways that lead to the determination of cell fates.

The developing *Drosophila* retina has proved to be well suited for genetic, molecular, and cellular experiments aimed at understanding the mechanisms of signal transduction. The adult eye is made up of a simple array of ~800 20-cell units called ommatidia. Each ommatidium contains eight photoreceptor cells, R1–R8, as well as four lens-secreting cone cells and eight other accessory cells. The cells that make up each ommatidium are thought to be recruited by a series of local cell-cell interactions, with differentiating cells instructing their immediate neighbors to adopt particular fates.

The R7 photoreceptor is the last of the eight photoreceptors to be recruited to the developing ommatidium. The presumptive R7 cell appears to face a simple choice between two alternative cell fates: it will develop into an R7 photoreceptor if it receives a signal that is initiated by activation of the Sevenless protein-tyrosine kinase receptor; otherwise it will adopt a nonneuronal cone cell fate. During the past year, Dr. Rubin's group has made significant progress in understanding the inductive event that leads to the recruitment of the R7 photoreceptor by using genetic analysis to reveal aspects of the intracellular pathway that transduces the signal initiated by acti-

vation of the Sevenless receptor. Two features make the Sevenless pathway ideal for genetic approaches to understanding signaling by receptor tyrosine kinases. First, both the R7 cell and the Sevenless protein are dispensable for viability and fertility. Second, the functioning of this signaling pathway can be inferred from the presence of the R7 cell in a live, anesthetized fly.

Transmembrane tyrosine receptors play important roles in a variety of physiological and developmental processes. How the signals initiated by activation of such receptors effect changes in cell physiology are still poorly understood. The intracellular machinery used to interpret and implement the instruction(s) conveyed by the activation of tyrosine kinase receptors is being actively investigated in a wide variety of developing organisms and tissues using both genetic and biochemical approaches. Biochemical studies with mammalian protein-tyrosine kinase receptors have led to the identification of proteins that bind to or are phosphorylated by tyrosine kinases. Although some of these interactions suggest potential mechanisms of signal transmission, their role *in vivo* is still unclear.

Sensitizing the System—A Way to Identify Particular Roles of Widely Utilized Proteins

Given the widespread role of tyrosine kinases in development, it is likely that the vast majority of genes whose products act in the Sevenless-mediated signal transduction pathway would also act in ear-

lier processes and thus be essential for viability. Requiring a homozygous mutant fly to survive to adulthood, where the presence or absence of an R7 cell can be assessed, could therefore prevent the identification of mutations in genes that also act in signal transduction pathways essential for earlier developmental stages. To get around this problem, Dr. Michael Simon and others in Dr. Rubin's group created a situation in which Sevenless kinase activity was limiting and then screened for mutations that altered the strength of Sevenless signaling. They first carried out a systematic genetic screen for mutations that decrease the effectiveness of signaling by Sevenless by looking for dominant enhancers of *sevenless* mutations. By adjusting the temperature at which flies carrying a temperature-sensitive allele of *sevenless* were grown, they could adjust Sevenless kinase activity to a level barely above the threshold necessary for R7 cell formation. Small reductions in the abundance or activity of other elements of the pathway might then be expected to lower signal strength sufficiently to cause a *sevenless* phenotype in flies grown at this threshold temperature. This sensitivity allowed them to identify genes encoding putative downstream elements of the pathway by screening for genes in which inactivation of only one copy of the gene, which would be expected to reduce the level of gene product by half, resulted in the absence of the R7 cell. Since the other copy of the gene remained functional, they were able to identify these loci even though their functions were essential for viability. Seven genes were identified in this screen. The products of four of the seven loci also appear to be involved in transduction of signals from another tyrosine kinase receptor, the *Drosophila* homologue of the epidermal growth factor (EGF) receptor.

Ras Plays an Essential Role in the Sevenless Signal Transduction Pathway

One of the loci identified in these genetic screens corresponds to the *Ras1* gene. Several previous studies had suggested a role for Ras activity in tyrosine kinase function. The fact that a twofold decrease in the level of Ras1 protein can give a dramatic effect on signaling by Sevenless suggests that Ras also plays a critical role in the Sevenless signaling pathway. To ask whether Ras1 activation alone is sufficient for Sevenless-mediated signaling, Dr. Mark Fortini used *sevenless* gene regulatory sequences to express dominant activating *Ras1* alleles in those cells of the developing eye that normally express *sevenless*. Constitutive activation of the Sevenless receptor has been shown by others to result in transformation of cone cell precursors into supernumerary R7 cells.

Identical results were obtained with activated Ras1 under *sevenless* gene control, suggesting that Ras1 activation may be the primary consequence of ligand-induced Sevenless signaling during R7 cell determination.

Regulation of Ras Activity—Possible Links Between Sevenless and Ras

The activity of Ras proteins is regulated by bound guanine nucleotides: the GTP-bound state is active, while the GDP-bound state is inactive. The ratio of GTP:Ras to GDP:Ras is determined by two antagonistic reactions. An active GTP:Ras molecule is inactivated by the intrinsic GTPase activity of the Ras protein, a process that is greatly stimulated by RasGAP. An inactive GDP:Ras molecule is activated by the exchange of the bound GDP molecule for a GTP molecule, a reaction that is increased by guanine nucleotide exchange proteins.

One of the four loci, Sos, identified by Dr. Simon and his co-workers as decreasing signal transduction initiated by either the Sevenless or EGF receptors, encodes a protein with sequence similarity to known guanine nucleotide exchange proteins, suggesting a model in which the activation of the Sevenless kinase by ligand binding would stimulate the activity of the Sos protein. The activated Sos protein would then stimulate Ras1 protein activity by promoting the conversion of GDP:Ras1 to GTP:Ras1. Moreover, in a genetic screen for mutations that increase signal transduction, Drs. Graeme Mardon and Ulrike Gaul isolated mutations in a gene, *Gap1*, that encodes a protein with sequence similarity to mammalian RasGAP. Their genetic analysis indicates that *Gap1* acts as a negative regulator of R7 cell determination, because loss of *Gap1*^{+} activity leads to the formation of supernumerary R7 cells, and reduction in *Gap1*^{+} activity increases the effectiveness of signaling by Sevenless.

Biochemical studies will be required to determine whether Sos or Gap1 protein activity is actually regulated by Sevenless activity. The available data do, however, permit the conclusion that the level of either Sos or Gap1 protein activity can be a limiting step in the decision by the presumptive R7 cell to become an R7 cell. The expression of Gap1 is spatially highly restricted, whereas that of Ras1 is not. The cell-type-specific expression and function of Gap1 is in contrast to the widespread expression and function of Ras1 and therefore suggests that distinct GTPase-activating proteins may regulate Ras1 in different developmental pathways.

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MOLECULAR MECHANISM OF TRANSMEMBRANE SIGNAL TRANSDUCTION BY G PROTEIN–COUPLED RECEPTORS

THOMAS P. SAKMAR, M.D., *Assistant Investigator*

The visual proteins transducin and rhodopsin are members of a superfamily of related G proteins and G protein–coupled receptors. Light-activated rhodopsin catalyzes guanine nucleotide exchange by transducin, which ultimately leads to a change in membrane cation conductance in the rod cell and a neural signal. The visual system provides an excellent model to study the molecular mechanism of transmembrane signal transduction by G protein–coupled receptors.

Current research interests in Dr. Sakmar's laboratory focus on structure-function relationships in rhodopsin and transducin. Under investigation are the ground state structure of rhodopsin, the interactions between specific amino acid residues and the 11-*cis*-retinal chromophore that control spectral

properties and photochemistry, the mechanism for transmitting a photochemical signal from the core of the receptor to the surface, and the specific domains on the cytoplasmic surface that bind and activate transducin.

The basic approach of the laboratory is to reconstitute heterologously expressed rhodopsin and transducin in defined *in vitro* systems. A multifaceted approach using a variety of complementary biochemical, biophysical, and spectroscopic methods is employed to probe site-directed mutants.

Spectroscopic Studies of Mutant Visual Pigments

A central question in vision research involves the mechanism of visual pigment spectral tuning. Nearly all vertebrate visual pigments share a common chromophore, 11-*cis*-retinal. However, the absorption maxima values of visual pigments range from near-ultraviolet to red. These spectral differences must be represented in unique chromophore-opsin interactions.

In humans the differences in absorption maxima of the cone pigments that underlie human red-green color vision must result from differences in the amino acid sequences of the respective opsin proteins. Fifteen amino acid substitutions distinguish the human green pigment (530 nm) from the red (560 nm). Three of these residues were suggested to produce this spectral difference through a genetic analysis of eight primate visual pigments. The amino acid at each of these three positions in the rod pigment rhodopsin (500 nm) matches that of the green pigment.

The influence of these residues was tested experimentally in Dr. Sakmar's laboratory by substituting the amino acid residues of the red pigment into rhodopsin. The spectral properties of a series of mutant pigments in which hydroxyl-bearing amino acids were introduced indicated that two of the three positions in combination appear to account for about three-quarters of the absorption difference between the human green and red pigments. Thus tyrosine 277 and threonine 285 appear to be involved primarily in red-green spectral tuning. However, other amino acid residues, including a serine at position 180, are likely to contribute to lesser degrees.

In rhodopsin, spectral tuning was shown not to be influenced by electrostatic interaction with carboxylates other than the Schiff base counterion. A neutral chromophore-binding pocket model in which dipole and hydrogen bonding interactions predominate has been proposed for rhodopsin. A similar model is likely to apply to the green and red color pigments as well. A complete understanding of spectral tuning in the visual pigments will require detailed spectroscopic studies, including resonance Raman spectroscopy of mutant rhodopsins.

Resonance Raman vibrational spectroscopy has been an important tool for studying the structures of the chromophores of visual pigments. In collaboration with Dr. Richard Mathies and Steven Lin, the laboratory developed a microprobe system to allow resonance Raman spectroscopy of microgram quantities of recombinant visual pigments. This technique was employed to study the effects of substitutions of carboxylic acid groups in the third transmembrane helix of rhodopsin. The results confirmed and supplemented the earlier observations concerning the role of glutamic acid 113 in rhodopsin that acts to stabilize the positive charge of the protonated Schiff base chromophore linkage. Based on the structural information obtained in these studies, a model of the chromophore-binding pocket of rhodopsin was proposed that will be used to direct further studies into the mechanism of wavelength regulation by visual pigments.

Fourier-transform infrared (FT-IR) spectroscopy is a novel vibrational-difference spectroscopic technique that provides specific structural information about the molecular changes that are associated with the photoactivation of rhodopsin. In collaboration with the laboratory of Dr. Friedrich Siebert, FT-IR spectra have been obtained on a collection of rhodopsin mutants with substitutions of carboxylic acid groups. The FT-IR technique is extremely sensitive to protonations and deprotonations of membrane-embedded carboxylic acid groups of rhodopsin that occur during its photobleaching pathway. Drs. Sakmar and Siebert hope to use a combination of site-directed mutagenesis and FT-IR spectroscopy to follow intramolecular proton transfers that accompany the formation of the active state of rhodopsin.

Rhodopsin-Transducin Interactions

Light-activated rhodopsin catalyzes guanine nucleotide exchange by transducin. It was previously shown by biochemical and flash photolysis studies of site-directed rhodopsin mutants that a highly conserved glutamic acid–arginine sequence in rhodopsin was involved in transducin binding. The second and third cytoplasmic loops of rhodopsin were shown to be necessary to activate bound transducin. Dr. Sakmar's laboratory is interested in identifying specific domains of rhodopsin and transducin involved in these discrete binding and activation events.

To facilitate the quantitative assay of rhodopsin-transducin interactions, Dr. Karim Fahmy has devel-

oped a spectrofluorimetric method designed to allow simultaneous illumination and excitation-emission fluorescence measurements of mixtures of recombinant proteins. Rhodopsin-catalyzed binding of GTP or a GTP analogue to transducin results in a large increase in its intrinsic fluorescence. Mixtures of transducin and rhodopsin can be assayed by this method to determine the kinetic rate constants of their interaction and to evaluate the specific effects of mutations.

Studies of a series of site-directed mutants of rhodopsin with alterations in their cytoplasmic domains have been performed. The eventual aim of this approach is to reconstitute purified recombinant rhodopsin and transducin under defined conditions where specific physical measurements relating to their interaction can be obtained.

Dr. Sakmar is also Assistant Professor and Head of Laboratory at the Rockefeller University.

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DEVELOPMENT AND FUNCTION OF THE SYNAPSE

Richard H. Scheller, Ph.D., *Associate Investigator*

The nervous system is composed of large numbers of unique cells that communicate with each other via the regulated release of chemical neurotransmitters. These synaptic interactions govern animal behavior. Modulation of the efficacy of synaptic communication is thought to underlie learning and memory. Dr. Scheller and his colleagues are interested in understanding the molecular mechanisms of synaptic formation during development and regeneration in the peripheral nervous system after nerve injury. It is also a goal to contribute to an understanding of how the nerve terminal functions in regulating release of neurotransmitters.

Synapse Development

Motor neurons in the spinal cord send axons to muscle fibers throughout the body. When axons contact muscle fibers, a highly ordered structure consisting of a presynaptic nerve terminal and postsynaptic site develops. The postsynaptic element is made up of a membrane rich in receptors for the neurotransmitter and an indentation in the membrane called the junctional fold. One key event in the development of the neuromuscular junction is the redistribution of neurotransmitter receptors that occurs when nerve contacts muscle. Initially receptors for the neurotransmitter, in this case acetylcholine, are randomly distributed on the muscle fiber. When the nerve contacts muscle, neurotransmitter receptors aggregate underneath the nerve terminal in an appropriate position to detect the chemicals released during synaptic transmission.

Agrin is a component of the extracellular matrix that causes acetylcholine receptors to cluster when added to muscle fibers growing in culture. Dr. Scheller and his colleagues have isolated recombinant DNA clones encoding agrin molecules and through an analysis of the nucleotide sequence defined the primary amino acid sequence of the molecule. Comparison of the predicted agrin sequence to the proteins in the data bank revealed similarities to two domains of laminin, kazal-type protease inhibitors, epidermal growth factor (EGF) repeats, serine/threonine-rich regions, and internal repeats. Analysis of the genomic sequence dem-

onstrates that the protease inhibitors are each encoded on separate exons, as are the serine/threonine-rich domains. The EGF repeats are flanked on the carboxyl terminus by introns. The region of similarity to laminin domain III is flanked by introns on both ends and contains a single intervening sequence within the domain. This intron is in a similar position to that in authentic laminin, consistent with the hypothesis that this gene evolved from a pool of ancestral exons now found in a variety of other molecules.

A variety of agrin proteins are synthesized from a single gene by alternative RNA splicing. Three regions of the protein make use of alternative splicing, resulting in 16 possible forms of the molecule. At amino acid position 1779, the alternate use of two exons gives rise to four possible splicing patterns. The result is a set of agrin molecules with 0, 8, 11, or 19 (8 + 11) amino acid inserts at this position. As a first step in understanding the physiological roles of the alternate splicing patterns, Dr. Scheller and his colleagues expressed the four forms of agrin in CHO and COS cells. The transfected cells were cocultured with a variety of muscle cells, including primary cells, C2-derived myotubes, or S27-derived myotubes. C2 myoblasts are cells that, when grown at the appropriate density in low serum, fuse to form myotubes. S27 cells are derivatives of C2 cells, selected because they do not synthesize proteoglycans. The S27 cells generate a lower level of spontaneous acetylcholine receptor (AChR) clusters than do the C2 cells. All forms of agrin are able to generate clusters of AChR when cocultured with either primary or C2-derived myotubes. In contrast, only the forms of agrin containing the 8–amino acid insert (the 8– and 19–amino acid insert forms) were active in generating clusters on S27-derived myotubes. Fluorescence-activated cell-sorting (FACS) analysis clearly demonstrates that this is not due to different levels of agrin on the surface of the transfected cells.

From these data, Dr. Scheller and his colleagues conclude that agrin may cluster receptors by two independent mechanisms—one that requires proteoglycans and another that is independent of proteoglycans but requires the 8–amino acid exon. The developmental expression of these splicing patterns is currently being investigated. It is possible that the introduction of different exon sequences at various times in development regulates different phases of development of the neuromuscular junction.

Mechanisms of Synaptic Transmission

When the action potential travels down the nerve and enters a release zone, changes in the membrane potential open channels that allow calcium to enter the cell. The calcium promotes transmitter release and membrane fusion. The membrane then recycles, forming new vesicles that are then replenished with chemical transmitter. This cycle might be considered the fundamental process that underlies nervous system function, yet little is known about the molecular mechanisms involved. In an attempt to define the molecular mechanisms that regulate membrane flow in the nerve, Dr. Scheller and his colleagues have begun to characterize the proteins associated with the critical organelle in the process, the synaptic vesicle.

Synaptotagmin, or p65, contains a membrane anchor and two regions homologous to protein kinase C (PKC). This homology is in the C2 region of the regulatory domain of PKC. This region is a feature of PKC isoforms that translocate to the membrane as part of their activation process and is found in other molecules, such as PLA2, which also translocate as part of their activation process. The laboratory prepared synaptosomes, solubilized the membranes in various detergents, and immunoprecipitated with p65 antibodies. A set of 35-kDa proteins are immunoprecipitated under all conditions tested. Further characterization of these molecules, called syntaxins, has resulted in the following model. Dr. Scheller and his colleagues propose that syntaxins are localized to the presynaptic plasma membrane via a carboxyl-terminal hydrophobic membrane anchor. The data further suggest that the syntaxins associate with both synaptic vesicle–anchored p65 and the N-type calcium channel. These interactions are therefore proposed to be involved in docking synaptic vesicles at active zones.

In the early 1980s, Dr. Regis Kelly and his coworkers isolated a set of monoclonal antibodies that specifically recognized proteins on synaptic vesicles. One of these antibodies recognizes an antigen, SV2, which is widely distributed throughout the nervous system and is highly conserved between species. To characterize SV2 further, Dr. Scheller and his colleagues obtained amino acid sequence and isolated cDNAs encoding the protein. The predicted SV2 amino acid sequence has 12 hydrophobic domains and significant amino acid sequence identity with bacterial sugar and drug transporters. The 12-membrane spanning organization is characteristic of transporters. The mammalian plasma membrane molecules use the Na^+ gradient as an energy source for the transport. In contrast, the bacterial transporters use an H^+ gradient, as do synaptic vesicles. The laboratory is currently characterizing other members of the SV2 family and investigating the function of the protein.

The project described in this section is funded in

part by a grant from the National Institute of Mental Health.

Dr. Scheller is also Associate Professor of Molecular and Cellular Physiology and Associate Professor of Biological Sciences (by courtesy) at Stanford University School of Medicine.

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COMPUTATIONAL NEUROBIOLOGY OF THE VISUAL CORTEX

Terrence J. Sejnowski, Ph.D., *Investigator*

The visual system is the best-understood sensory system in the mammalian brain, and the visual cortex is the most intensively studied visual area. There is growing evidence that the development of the visual cortex depends on electrical activity driven by the retina. The self-organization of neurons into cortical columns provides the brain with populations of neurons having a wide variety of response preferences. Dr. Sejnowski's laboratory is using computer models constrained by the response properties of neurons from single-cell recordings and psychophysical measurements on performance to provide a conceptual framework for how visual information is distributed in neural populations. Such models are being used to explore how the visual cortex represents the three-dimensional world, how this representation may arise during development, and how the information coded by these neurons might be used to coordinate actions such as eye movements.

Disparity Selectivity in Visual Cortex

When eyes are fixed on a location in space, the slight positional shift in the projection of an object between the two eyes, called the retinal disparity, provides information about the distance of the object from the point of fixation. Neurons in the first cortical stage of vision are sensitive to disparity, and the development of their disparity selectivity depends on binocular vision during a critical period in early life. In the adult visual cortex, neurons are observed to be either dominated by input from one eye (monocular cells) or relatively balanced with input from both eyes (binocular cells). Furthermore, binocular cells tend to prefer zero disparity, whereas relatively monocular cells tend to prefer nonzero disparities. Computer models have been used to study how mechanisms that have been uncovered for plasticity in visual cortex during development could account for observed relationships such as that between ocular dominance and disparity.

Drs. Michael Stryker and Kenneth Miller (University of California, San Francisco), using a computer model, have shown how the activity-based segregation of thalamic afferents in layer 4 of visual cortex could be driven by competition between inputs from the two eyes. Dr. Sejnowski's laboratory has extended this model to the development of disparity

selectivity by including correlations in the electrical activity between eyes. Corresponding points in the two eyes tend to be correlated because, on average, they look at the same point in space. The development of disparity selectivity was simulated in two stages: prenatal, when the two retinas have essentially independent activities, and postnatal, when the eyes are open and have correlated activities. By varying the degree of development that occurred in the model before eye opening, a mixture of monocular and binocular cells arose with the experimentally observed relationship to disparity.

Disparity can be combined with other cues to provide information about the distance of an object from the viewer. With grant support from the Office of Naval Research, Dr. Sejnowski's laboratory has shown how the vergence of the two eyes (angle between the two lines of sight) and the binocular disparity could be combined to represent the distance to an object in a population of neurons. Single neurons in the visual cortex have disparity-tuning curves that are broad and overlapping. In the network model, distances were represented by a population of such neurons whose responses were modulated by the vergence angle. Neurons with such properties have recently been observed in the primary visual cortex and the posterior parietal cortex, a region of the brain that is essential for the internal representation of external space. These new findings suggest that transformations from retinal to spatial representations could be initiated much earlier than previously thought in the visual system. Computer models are being developed to study the consequences of incremental spatial transformations in a feedforward hierarchy of cortical maps.

Motion Processing in Visual Cortex

The primate visual system is very good at tasks such as tracking a moving object against a textured background. To track a moving object, the visual system must integrate local motion estimates from many neurons, each with a limited spatial receptive field. The integration of information on motion can be affected by a variety of cues such as contrast, spatial frequency, binocular disparity, color, transparency, and occlusion. Thus the integration of motion signals cannot be performed in a fixed manner but must be a dynamic process dependent on the properties of the visual stimulus. There is the additional problem of segregating information from a single object when there are several objects.

Dr. Sejnowski's laboratory has developed a simple model for motion processing in area MT, a region of the primate visual cortex that specializes in representing motion. The model assumes two popula-

tions of neurons at each position in the visual field: one population computes estimates of motion in a local region of the visual field while the second population estimates the relevance or reliability of each local motion estimate. Outputs from the second population of neurons then gate the outputs from the first population of neurons through a gain-control mechanism, before the local motion estimates are integrated to form more global estimates. The proposed mechanism of gain control is consistent with measured responses of cortical cells under conditions of interfering motion of transparent stimuli. In addition, predictions were made for the response properties of neurons in MT that may be estimating the reliability of the local motion estimates.

Visual area MT provides the oculomotor system with information about the motion of moving objects so that they can be smoothly tracked by eye movements. Dr. Sejnowski is collaborating with Dr. Stephen Lisberger (University of California, San Francisco) to develop models of the oculomotor system that will complement the models of visual processing. Their models of motor control are based on networks of neurons that include feedback connections, which makes them highly dynamic.

Dr. Sejnowski is also Professor in the Computational Neurobiology Laboratory at the Salk Institute for Biological Studies and Professor of Biology and Adjunct Professor of Neuroscience, Physics, Psychology, Cognitive Science, Electrical and Computer Engineering, and Computer Science and Engineering at the University of California, San Diego.

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DIVERSE ROLES OF CYCLIC NUCLEOTIDES IN NEURONAL SIGNALING

STEVEN A. SIEGELBAUM, PH.D., *Associate Investigator*

Cyclic nucleotides play a number of important roles in neuronal signaling. The first well-characterized action of cAMP in neurons was as a second messenger for certain modulatory transmitter actions. Here the binding of transmitter to its membrane receptor was shown to lead to an elevation of cAMP with a resultant activation of the cAMP-dependent protein kinase, leading to an increase in protein phosphorylation. Phosphorylation of ion channels was shown to underlie the modulatory changes in neuronal electrical activity. Transmitter actions mediated by this cAMP cascade were generally characterized by their relatively slow time course, lasting many seconds to several minutes. Thus it was somewhat surprising when it was shown that cyclic nucleotides also mediate more-rapid forms of neuronal signaling associated with both visual and olfactory sensory signal transduction. Such rapid signaling roles for cyclic nucleotides depend on the direct activation of cyclic nucleotide–gated (CNG) ion channels through the binding of cGMP or cAMP to the channel. Over the past several years a number of laboratories have cloned related CNG channels from photoreceptor and olfactory receptor neurons. During the past year, research in Dr. Siegelbaum's laboratory has focused on the biophysical properties of a CNG channel from catfish olfactory neurons and the role of this channel in olfactory signal transduction.

The catfish olfactory CNG channel was cloned in collaboration with Dr. Richard Axel (HHMI, Columbia University), and the channel was then expressed in *Xenopus* oocytes. The genes for CNG channels from bovine photoreceptors as well as rat, bovine, and catfish olfactory neurons are highly homologous, with ~70% identity at the amino acid sequence level. As pointed out by Drs. Benjamin Kaupp and Shosaku Numa, these channels all contain a domain near the carboxyl terminus that is homologous to the cyclic nucleotide–binding domains of the cAMP- and cGMP-dependent protein kinases. Although gated by cyclic nucleotides, these channels show significant homology with the voltage-gated channel family and contain a putative

voltage sensor (S4) membrane-spanning domain and a membrane domain (H5 or SS1-SS2) thought to form the lining of the pore.

To test whether the CNG channels show any functional similarity to the voltage-gated channels, Dr. Siegelbaum and his colleagues have used single-channel recording to study the properties of the cloned CNG channel expressed in *Xenopus* oocytes. The channel is activated equally well by cAMP and cGMP with a $K_{1/2}$ for activation of ~ 50 μm and a Hill coefficient of 1.4, suggesting that the binding of two or more cyclic nucleotide molecules is required for activation. At the single-channel level, increasing concentrations of cyclic nucleotides lead to an increase in channel open probability and open burst duration. The single-channel current shows a main open conductance state of ~ 55 pS, with a prominent subconductance state of ~ 27 pS. Entry into this subconductance state depends on the binding of protons to an external site on the channel and is relieved by large depolarizations. This behavior is similar to that described by Dr. Peter Hess and his colleagues for the subconductance state of voltage-gated calcium channels. Patch-clamp studies of the catfish CNG channel in native olfactory neurons reveal similar properties. However, the native channel is 20-fold more sensitive to cyclic nucleotides ($K_{1/2}$ of 2–3 μM). Future experiments are planned to address this discrepancy.

Does the S4 region confer significant voltage dependence to channel gating? Dr. Siegelbaum and his colleagues find that the gating of the channel depends only weakly on membrane voltage, similar to the results of others. Channel open probability increases by a factor of two for a 100-mV depolarization. This weak voltage dependence is 10- to 20-fold less than the voltage dependence of typical voltage-gated channels. Apparently the S4 domain does not confer significant voltage dependence.

In other work, Dr. Siegelbaum and his colleagues are focusing on the role of the catfish CNG channel in olfactory adaptation. A prominent feature of olfactory signal transduction is that prolonged exposure to an odorant causes a relatively rapid decline (or adaptation) in the response of an olfactory neuron to that odorant. A rise in intracellular calcium has been proposed to be important in olfactory adaptation, although the mechanism whereby calcium reduces the response to an odorant is not known. Dr. Richard Kramer and Dr. Siegelbaum have studied the possibility that internal calcium may regulate the functioning of the olfactory CNG channel.

These studies were performed on CNG channels in membrane patches obtained from catfish olfactory neurons. Elevating internal calcium was found to have a profound inhibitory effect on the activation of these channels by cyclic nucleotides. Calcium was found to act by shifting the dose-response curve for channel activation to higher cyclic nucleotide concentrations without altering the maximal response. Moreover, this effect occurs at physiological levels of calcium. Half-maximal inhibition occurs at ~ 3 μM Ca^{2+}.

How does internal calcium inhibit the activation of the CNG channel? A series of pharmacological experiments showed that this inhibitory effect does not depend on activation of a phosphodiesterase, protein kinases, protein phosphatases, or on calmodulin. However, the inhibitory action of calcium also does not appear to result from a direct action of calcium on the CNG channel, because the effect gradually washes out over 15–30 min after a patch is excised from an olfactory neuron. Moreover, the cloned CNG channel expressed in *Xenopus* oocytes does not exhibit the calcium inhibition. Thus it was concluded that calcium acts on an accessory protein that is associated with the CNG channel in olfactory neuron membranes.

Thus the olfactory system provides a useful model for studying neuronal signal transduction and neuronal plasticity. Studies on the molecular bases of these phenomena should provide insight into many of the basic mechanisms controlling nerve cell behavior.

Dr. Siegelbaum is also Associate Professor of Pharmacology in the Center for Neurobiology and Behavior at Columbia University College of Physicians and Surgeons.

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PATTERN FORMATION AND NEURONAL CELL RECOGNITION IN THE *DROSOPHILA* VISUAL SYSTEM

HERMANN STELLER, PH.D., *Assistant Investigator*

The overall objective of Dr. Steller's research is to understand how functional neuronal circuits are established and maintained during development. He and his co-workers are currently focusing on three major areas.

Axon Guidance and Neuronal Cell Recognition

Dr. Steller's group is studying two different stages of visual system development to investigate the cellular and molecular mechanisms by which axons find and recognize their proper synaptic partners. The *Drosophila* eye consists of ~800 repeating units, called ommatidia. Each ommatidium contains eight photoreceptor neurons that represent three major cell types projecting to different target cells in the optic ganglia. The major class of photoreceptors, R1–6, sends axons to the first optic ganglion, the lamina. Photoreceptor axons from R7 and R8 project deeper into the brain, to different regions of the medulla. The growth cones of these axons must navigate over a long distance and make a number of highly specific choices. The goal of this research is to understand what signals guide axons to their destinations and how these signals are generated, received, and interpreted.

Dr. Steller's group has discovered that retinal axon fascicles can make at least some of their pathfinding decisions independently of other fascicles, suggesting that they rely on positional guidance cues to establish proper retinotopic maps. Position-specific differences between photoreceptor cells have been revealed by enhancer trap lines that produce a gradient of reporter gene expression along the dorsoventral axis of the developing retina. The gene corresponding to one of these gradient lines has been isolated, and mutations in this gene have been obtained that result in embryonic lethality. The functional and molecular characterization of this locus is in progress.

Dr. Steller and his colleagues have also screened for mutations that perturb the projection pattern of photoreceptor cells at very early developmental stages, when axons enter the brain. A number of mutants with severely abnormal patterns of axon ingrowth have been isolated, and the developmental, genetic, and molecular characterization of this material has been initiated.

Dr. Steller's group is also using the optic nerve of the *Drosophila* larva as a simple model system to investigate how specific neuronal connections are established. They previously identified a gene, *disconnected* (*disco*), that is required for establishing stable connections between the larval optic nerve and its target cells in the developing brain. The *disco* gene has been cloned, and its structure, nucleotide sequence, and pattern of expression have been determined.

These studies suggest that *disco* encodes a transcription factor with autoregulatory properties. Consistent with such a function is the group's observation that the *disco* protein has sequence-specific DNA-binding activity *in vitro* and that two high-affinity binding sites are located very close to the *disco* transcription unit. Ectopic expression of *disco* protein under an inducible promoter in transgenic flies results in severe developmental defects and embryonic lethality. These defects include both a drastic reduction of the axon scaffold and connectivity defects in both the peripheral and central nervous systems. Experiments are in progress to test the idea that *disco* controls the activity of genes required for the establishment of stable connections between the larval optic nerve and its target cells. (This work was supported by a grant from the National Institutes of Health.)

Role of Innervation for Neurogenesis and Survival of Target Cells

It has been noticed for many years that synaptic input can have a profound influence on the fate and differentiation of target cells. In *Drosophila*, the proper development of the adult optic ganglia de-

pends on innervation from the eye. In the absence of retinal innervation, adult flies entirely lack the first optic ganglion, the lamina, which receives direct synaptic input from the outer photoreceptor cells R1–6.

Dr. Steller's group has found that the birth of lamina neurons is controlled by innervation from the developing eye. The neurons are produced by a wave of mitotic activity induced by the arrival of photoreceptor axons in the brain. These results suggest a novel mechanism for matching the number of target neurons in the first optic ganglion to the number of incoming photoreceptor axons, and they explain how developmental synchrony between the *Drosophila* retina and the first optic ganglion is achieved.

More recently Dr. Steller and his colleagues have found that the differentiation, but not the birth, of glial cells in the lamina depends on retinal innervation. Several different approaches are now being used to elucidate the detailed cellular and molecular mechanisms underlying this process.

Although the importance of retinal innervation on the development of the adult optic ganglia of *Drosophila* is well documented, little is known about retrograde effects of the brain on photoreceptor cells in the compound eye. Dr. Steller and his colleagues have recently discovered the first evidence for the existence of such retrograde effects in the *Drosophila* visual system. Although photoreceptor cells develop normally in the absence of connections to the optic ganglia, their continued survival requires these connections. This situation is reminiscent of trophic interactions that are commonly found in invertebrates.

Genetic Control of Cell Death

Dr. Steller's group is interested in isolating genes required for the initiation or execution of pro-

grammed cell death in *Drosophila*. They have found that the ultrastructural characteristics of cell deaths seen in the *Drosophila* embryo are strikingly similar to apoptotic deaths described in mammalian systems. Techniques have been developed that permit the rapid and reliable visualization of apoptotic cells in live embryos.

These methods have been used to screen for cell death–defective mutants. A complex genetic locus on the third chromosome is required for either the commitment to, or the execution of, a cell death program. The DNA encompassing this locus has been cloned, and the molecular characterization of this interval is in progress.

Dr. Steller is also Associate Professor of Neurobiology at the Massachusetts Institute of Technology and Adjunct Assistant Neurobiologist at Massachusetts General Hospital, Boston.

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STUDIES ON A MOUSE MUTANT SUPPORT THE CONNECTION BETWEEN LONG-TERM POTENTIATION AND LEARNING

Charles F. Stevens, M.D., Ph.D., *Investigator*

Most neurobiologists have accepted the notion that long-term potentiation (LTP)—the enduring increase in synaptic strength that occurs with certain types of synaptic use—is the cellular basis for storing memories in the hippocampus. This acceptance is based mainly on three lines of evidence: first, LTP has the properties required for a memory mecha-

nism (it is associative, synapse specific, rapidly established, and long lasting); second, LTP is prominent in the hippocampus, a brain region known to be involved in storing memories; and third, several drugs that block LTP, by interfering with the function of *N*-methyl-D-aspartate (NMDA) receptors (APV [aminophosphono valerate], for example),

also block the spatial learning in rodents that lesion studies have associated with hippocampal function. An additional reason LTP has been so widely accepted as a memory mechanism is that there are no alternatives.

Despite the wide acceptance of LTP as a cellular substrate for memory, the actual evidence in favor of this mechanism is inconclusive. The most direct evidence comes from the experiments, noted above, in which NMDA receptor function is blocked with drugs like APV. But APV and similar agents also block the slow component of synaptic currents, and studies in many brain areas have demonstrated that this slow component is often required for computations. An alternative explanation of the NMDA-blocking results, then, would be that APV prevents proper functioning of hippocampal circuits so they cannot perform some computation required for learning to be established by a mechanism entirely independent of LTP. How, then, might LTP be used in the hippocampus? Perhaps LTP modifies hippocampal circuits so they can compute more efficiently. LTP would thus be producing activity-dependent circuit modifications like those that result in ocular dominance columns in area 17, but modifications that are constantly updated.

On this view, LTP would be used to tune up hippocampal circuits continually so they could carry out some computation associated with forming memories, but would not be the storage mechanism itself.

Because blocking NMDA receptors simultaneously prevents the triggering of LTP and interferes with synaptic transmission, the only way to test the hypothesis that LTP is a memory storage mechanism is to find ways of preventing LTP that do not involve modifications of synaptic transmission. Dr. Alcino Silva, in the laboratory of Dr. Susumu Tonegawa (HHMI, Massachusetts Institute of Technology), has produced a mutant mouse that lacks the α subunit of calcium/calmodulin kinase type II (CaM kinase II), an enzyme that pharmacological studies have implicated in some step of the production of LTP. The brains of these mutant mice, and their behavior, are grossly normal. The Stevens laboratory, in collaboration with Dr. Tonegawa, has found, however, that the mice are deficient in LTP production but that their synaptic transmission is intact. Behavioral work, done in collaboration by the laboratories of Dr. Tonegawa and Dr. Jeanne Wehner, finds that the mutant mice are also deficient in spatial learning. These studies thus strengthen the hypothesis that LTP is a memory mechanism.

Field potential recordings survey a very large population of neurons and thus are useful in assessing the extent to which some particular manipulation interferes with the triggering of LTP. Such field potential recording revealed, using a standard procedure that almost always produces LTP in the littermate controls, that the mutant mice only rarely exhibit LTP.

The advantage of field potential recordings is that the properties of very large populations of neurons are surveyed. The difficulty with the technique is that the triggering of LTP is known to depend on the neuron's depolarization, which in turn results from the net effect of the synaptic input. If the synaptic input is insufficient for any reason at all, LTP would be diminished simply because the stimulus did not produce an adequate depolarization. A more sensitive test for LTP, then, is to use whole-cell recording and control the neuron's membrane potential by voltage clamp. This method examines one by one (rather than revealing population properties) but is very sensitive in that stimulation of just one or a few boutons can produce LTP. The Stevens laboratory thus studied neurons from mutant mice and from normal littermates; again, they found that LTP is greatly diminished. From both field potentials and whole-cell recordings, then, the mutant animals appear to be deficient in LTP, but some fraction of the neurons do show what appears to be normal, if small, LTP.

LTP is triggered by the calcium influx through NMDA receptor channels. The mutant mice might, through either a developmental or regulatory mechanism that depends on the activity of CaM kinase II, lack effective NMDA receptor function. To examine this question, Dr. Stevens and his colleagues compared the behavior of NMDA receptor channels in hippocampal neurons from mutant and normal animals. They find that the amplitude and time course of the NMDA component of synaptic currents appears not to differ between normal and mutant mice. NMDA receptor channels have a special property: the fraction of the time the channel-ligand complex spends in a conducting state depends on the neuron's voltage. The voltage dependence is known, in some circumstances, to be modified by phosphorylation. The Stevens laboratory compared this voltage dependence in mutant and controlled mice and found this property of NMDA receptors to be unaffected in the mutant animals.

In summary, postsynaptic mechanisms are intact in the mutant mice, so hippocampal computations should be performed normally. Nevertheless, both LTP and spatial learning are deficient. These observations strengthen the hypothesis that LTP is indeed a memory mechanism, but a final interpretation of the results must await studies that identify the precise mechanism underlying the defects.

Dr. Stevens is also Professor at the Salk Institute for Biological Studies and Adjunct Professor of Pharmacology at the University of California School of Medicine, San Diego.

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CELL AND BODY PATTERNING IN *DROSOPHILA*

GARY STRUHL, PH.D., *Associate Investigator*

Dr. Struhl's research is focused on the molecular nature and mode of action of spatial determinants responsible for organizing cell and body patterns in *Drosophila*. The basic body pattern of head, thoracic, and abdominal segments is prefigured at fertilization by distinct anterior, posterior, and terminal determinant systems laid down in the egg during oogenesis. All three systems operate in the early, syncytial embryo in which signaling molecules can diffuse from one region to another through a common cytoplasm. Later, spatial signals must be locally generated in some cells and passed to others. The roles of the three early determinant systems, as well as the molecular mechanisms involved in their establishment and function, are under investigation. A general method has also been developed for analyzing the roles of putative signaling molecules in later, cellular systems.

Early Determinant Systems

Prior work by Dr. Christiane Nüsslein-Volhard and her colleagues has established that two morphogens, *bicoid* (*bcd*) and *nanos* (*nos*), are responsible for specifying most aspects of anteroposterior body pattern. Recent studies in Dr. Struhl's laboratory have established that *bcd* and *nos* act at least in part by blocking the accumulation of protein from uniformly distributed maternal transcripts of the *caudal* (*cad*) and *hunchback* (*hb*) genes. The involvement of *bcd* in regulating *cad* protein expression is interesting because *bcd* is a homeodomain protein, and previous work in Dr. Struhl's laboratory has established 1) that it can directly bind and transcriptionally activate subordinate regulatory genes and 2) that single–amino acid changes in the homeodomain that alter DNA-binding specificity also prevent the regulation of *cad* protein expression. Hence it is possible that *bcd* may bind and translationally regulate *cad* mRNA via the homeodomain. For *nos*, recent experiments of Drs. Struhl, Robin Wharton, and Peter Lawrence have established 1) that the sole role of *nos* in generating posterior body pattern is its regulation of *hb* protein expression, 2) that this regulation depends critically on small cis-acting sequences in the *hb* transcript, and 3) that the resulting gradient of *hb* protein, a zinc finger transcription factor, specifies posterior body pattern by activating or repressing downstream regulatory genes in a concentration-dependent fashion. Thus concentration-dependent binding of *hb* mRNA

by *nos* protein may determine the profile of the *hb* protein gradient and thereby govern posterior body pattern. For both the *bcd-cad* and *nos-bcd* interactions, current experiments are in progress to define further the cis-acting target sites, to assay for direct binding, and to determine the molecular mechanism of translational repression.

A third determinant system is responsible for specifying the anterior and posterior ends of the body. This terminal system depends critically on the activity of *torso* (*tor*), a receptor tyrosine kinase. Prior experiments of Drs. Jordi Casanova and Struhl have indicated that *tor* protein functions as a ubiquitous surface receptor that is activated in the vicinity of the poles in response to a localized ligand. More-recent experiments have suggested that when the receptor concentration is abnormally low, the ligand is not efficiently trapped at the poles but can diffuse to more-central portions of the body, where it activates the *tor* receptor ectopically. The degree of ectopic activation observed under this unusual circumstance appears to depend on the dosage of a second gene, *trunk* (*trk*), which is critically required for normal activation of the *tor* receptor. Drs. Casanova and Struhl have recently determined the sequence of the *trk* gene, which may encode a small, secreted protein. Experiments to test the role of the *trk* protein, particularly the possibility that it encodes the *tor* ligand, are in progress.

Patterning in Cellular Systems

Early *Drosophila* embryos differ from most other patterning systems because spatial signals arise and diffuse within a common cytoplasm rather than in populations of cells comprising organs or appendages. However, few if any spatial signaling molecules have been identified in such cellular systems, nor are their roles well described or understood.

One promising candidate for a diffusible factor that may govern cell pattern is the protein product of the *Drosophila* segment polarity gene *wingless* (*wg*). It appears that *wg* encodes a secreted protein expressed in discrete subsets of cells in each embryonic segment and in each imaginal disc giving rise to an adult appendage. In the embryo, *wg* activity in some cells is required for maintaining the expression of the *engrailed* gene in neighboring cells. Moreover, mutations that reduce or eliminate *wg*

activity have a profound effect on global cell pattern, both in embryonic segments and in the adult appendages. These findings have raised the possibility that *wg* protein expressed by some cells may be responsible for organizing the pattern of surrounding cells.

Drs. Struhl and Konrad Basler have devised a new and general method for controlling where genes are expressed in developing tissues to test whether cells expressing *wg* protein can organize the pattern of neighboring, nonexpressing cells. The gist of this technique is to use a site-specific recombinase (the yeast protein encoded by the *flp* gene) to fuse the promoter of one gene to the coding sequence of another. This technology has been used to activate heritably the *wg* coding sequence in randomly positioned cells during development. Ectopic activation of *wg* in dorsally positioned cells of the leg imaginal discs can reorganize the pattern of surrounding cells, causing dorsoventral and proximodistal pattern duplications, including the formation of supernumerary limbs. The expression of *wg* protein in a small subset of cells can therefore exert a profound influence on the developmental behavior of surrounding cells, indicating that these cells are equivalent to classical embryonic organizers. Furthermore, the nature of the duplicated patterns as well as the restricted positions in which they arise suggest the possibility that in normal leg discs ventrally positioned cells provide a source for *wg* protein, which spreads dorsally and functions as a morphogen to control dorsoventral pattern in the limb.

Dr. Struhl is also Associate Professor of Genetics and Development and a member of the Center for Neurobiology and Behavior at Columbia University College of Physicians and Surgeons.

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STRUCTURE AND FUNCTION OF THE PRESYNAPTIC NERVE TERMINAL

Thomas C. Südhof, M.D., *Investigator*

Synapses are abundant neural structures (there are $\sim 10^5$ times more synapses in the mammalian brain than there are base pairs in the human genome) that constitute the principal point of information transfer between neurons. At the synapse, a presynaptic nerve terminal contacts a postsynaptic cell, probably via cell-cell contact proteins. Information is transferred between pre- and postsynaptic cells by the release of neurotransmitters and chemical signals from the presynaptic terminal, and the recognition of the neurotransmitters by the postsynaptic cell. In the presynaptic nerve terminal, neurotransmitters are stored at high concentrations in synaptic vesicles, specialized secretory organelles of the presynaptic nerve terminal, and released on synaptic vesicle exocytosis at the active zone of the presynaptic plasma membrane.

Over the past six years, Dr. Südhof and his colleagues have studied the nerve terminal by two approaches. First, the structures and functions of the proteins of synaptic vesicles, the central organelle in neurotransmitter release, were investigated. These studies have led to the molecular description of a number of synaptic vesicle proteins, accounting for a significant part of the total synaptic vesicle proteins, and to the formulation of the functional importance of some of these proteins. The work on synaptic vesicles has also allowed generalizations that may be applicable to other organelles. Second, Dr. Südhof's laboratory has initiated studies on the presynaptic plasma membrane and its interactions with synaptic vesicles during synaptic vesicle exocytosis/neurotransmitter release. These studies have led to the description of a family of synaptic cell surface proteins named neurexins that are related to the α-latrotoxin receptor, and to the discovery that the α-latrotoxin receptor as well as other neurexins directly interacts with synaptotagmin, a synaptic vesicle membrane protein.

With respect to synaptic vesicles, recent work in Dr. Südhof's laboratory has focused on two aspects: the structure and function of synaptotagmin, a major synaptic vesicle protein with a putative function in the Ca^{2+}-dependent exocytosis of synaptic vesicles, and the relation between the synaptic vesicle pathway and other cellular membrane trafficking events. These studies were carried out in collaboration with Dr. Reinhard Jahn (HHMI, Yale University).

Synaptotagmin is a $65,000\text{-}M_r$ protein that is present on synaptic vesicles in several differentially distributed isoforms (referred to as synaptotagmins I–

IV). Native synaptotagmin forms a high-molecular-weight complex, probably a homotetramer, on the synaptic vesicle membrane, to which it is attached by a single transmembrane region. The primary structure of synaptotagmin contains an internal repeat that is homologous to the C_2 domain of protein kinase C and is highly conserved from humans to fruit flies. Purified synaptotagmin I was shown to bind Ca^{2+} in a ternary complex together with negatively charged phospholipids. Ca^{2+} binding by synaptotagmin I was concentration dependent, with half-maximal binding observed at ~ 20 μM free Ca^{2+}, a concentration thought to be reached physiologically in the stimulated nerve terminal. These properties suggest that synaptotagmin may be the Ca^{2+} trigger on the synaptic vesicle surface that functions in Ca^{2+}-dependent exocytosis. In addition, evidence was obtained for a direct interaction of synaptotagmin I with neurexins and the α-latrotoxin receptor, which are thought to be synaptic plasma membrane proteins. These findings raised the possibility that synaptotagmin may also be involved in the docking of synaptic vesicles in the nerve terminal in addition to being a Ca^{2+} sensor.

The pathway of synaptic vesicles in the nerve terminal, although unique in terms of its speed and regulation, contains many similarities to ubiquitous cellular membrane trafficking pathways, in particular the receptor-mediated endocytosis pathway. In experiments performed several years ago in collaboration with Dr. Pietro De Camilli (HHMI, Yale University), Dr. Südhof's laboratory had shown that transfection of synaptophysin into fibroblasts results in the targeting of synaptophysin to a vesicular compartment that was identified as part of the receptor-mediated endocytosis pathway. Recent findings have shown that in developing neurons, synaptic vesicle proteins are present in a constitutively recycling pathway that also contains transferrin receptors. Only with neuronal maturation can a segregation of the pathways be seen. Furthermore, in endocrine cells both pathways seem to coexist. Finally, a new form of one of the synaptic vesicle proteins was recently discovered that, despite extensive homologies, was absent from synaptic vesicles but present in the receptor-mediated endocytosis pathway. Together these findings suggest that the receptor-mediated endocytosis pathway and the synaptic vesicle pathway are evolutionarily and mechanistically related.

Apart from these two central themes regarding

synaptic vesicles, the structures and functions of several other synaptic proteins are being studied in Dr. Südhof's laboratory, in particular rab3A and synapsins. Together these studies should contribute to the understanding of the molecular basis for nerve terminal function.

In a different approach, Dr. Südhof and his colleagues have studied the receptor for α-latrotoxin, a presynaptic spider toxin that causes massive neurotransmitter release from vertebrate nerve terminals. Purification of the receptor revealed it to be composed of a family of related high-molecular-weight subunits ranging from M_r 160,000 to 220,000 and a low-molecular-weight component of M_r 29,000. Cloning of these polypeptides demonstrated that the low-molecular-weight component represents a secreted protein with no sequence homology to current entries in the data banks.

The high-molecular-weight components, on the other hand, were discovered to be members of a polymorphic family of brain-specific cell surface receptors called neurexins. At least three genes for neurexins exist, each of which contains two independent promoters that specify transcripts whose coding region differs by more than 1,000 amino acids. Extensive alternative splicing creates a multitude of neurexins whose number could exceed 200 different proteins. Sequence analysis demonstrated that the neurexins contain a repeated sequence motif homologous to repeats found in several extracellular matrix proteins, in particular laminin A, agrin, slit, and perlecan. Most of these proteins have a well-characterized function in neuronal development and interact with cell surfaces, suggesting that the motif may represent a cell adhesion domain and that the neurexins may be cell adhesion receptors. The polymorphic nature of the neurexins together with the synaptic localization of at least some of them led Dr. Südhof and his colleagues to propose that they may represent synaptic cell recognition molecules involved in establishing synaptic specificity. A grant from the Perot Family Foundation provided support for the project described above.

A surprising result of the purification of the α-latrotoxin receptor was that a synaptic vesicle membrane protein, synaptotagmin, copurified with it. The suggestion raised by this observation, namely that the receptor might directly interact with synaptotagmin, was confirmed with affinity chromatography and other biochemical assays. Furthermore, when the cytoplasmic domains of different neurexins were tested for their ability to interact with synaptotagmin, they were also found to bind in a sequence-specific manner. These results suggest that synaptotagmins may target synaptic vesicles specifically to the active zone by interactions with the α-latrotoxin receptor and other neurexins, which would confer specificity to the otherwise nonspecific interactions of synaptotagmin with the phospholipid bilayer.

In work that is primarily funded by the National Institute of Mental Health, Dr. Südhof's laboratory has also investigated the role of inositol 1,4,5-trisphosphate (InsP$_3$) receptors in regulating intracellular Ca^{2+} concentrations in neurons. Neurons contain particularly high levels of InsP$_3$ receptors, and Dr. Südhof's laboratory discovered and molecularly cloned several novel InsP$_3$ receptors. In addition, structure-function relationships in InsP$_3$ receptors were explored, leading to the characterization of the ligand-binding domain in multiple InsP$_3$ receptors and to an elucidation of how Ca^{2+} regulates InsP$_3$ binding and InsP$_3$ receptor function. These studies will further the understanding of the dynamics of intracellular Ca^{2+} in neurons.

Dr. Südhof is also Professor of Molecular Genetics at the University of Texas Southwestern Medical Center at Dallas.

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MOLECULAR ENGINEERING APPLIED TO INTRACELLULAR SIGNAL TRANSDUCTION

Roger Y. Tsien, Ph.D., *Investigator*

Dr. Tsien's laboratory investigates intracellular signal transduction, with an emphasis on probing intact cells with carefully designed molecules that give optical readouts or controlled perturbations of internal biochemistry.

Signal Transduction to the Nucleus

Previous work from this laboratory showed that the catalytic subunit (C) of cAMP-dependent protein kinase moves reversibly into and out of the nucleus upon elevation and depression of cAMP levels. Because such translocation may be a key step in carrying cAMP signals into the nucleus to affect gene expression, Dr. Alec Harootunian investigated its mechanism to decide whether nuclear import and export of C are active processes, perhaps controlled by phosphorylation, or merely passive diffusion.

Covalent inactivation of the phosphorylating activity of C or co-injection of a competitive inhibitor of active nuclear transport did not prevent entry of REF-52 fibroblasts into the nucleus. Even when cAMP is high, exit of C from the nucleus could be demonstrated by increasing the laser power on the confocal microscope to photobleach the fluorescein label on the C that remains in the cytoplasm. Afterward, the unbleached C in the nucleus reequilibrated out into the cytoplasm to the same final concentration ratio and with the same kinetics as it had initially entered the nucleus. Therefore nuclear import and export appear to be passive diffusional processes once C is freed by the binding of cAMP to the regulatory subunit, which remains in the cytoplasm.

Studies of cAMP in *Aplysia* sensory neurons continued, with important controls to support the showier results obtained last year. Dr. Brian Bacskai developed a method to calibrate the fluorescein- and rhodamine-labeled cAMP-dependent protein kinase in terms of absolute cAMP concentrations. The enzyme was placed in a microdialysis capillary and imaged on the confocal microscope while the capillary was immersed in various cAMP concentrations. In collaboration with Dr. Beni Hochner from the laboratory of Dr. Eric Kandel (HHMI, Columbia University), the basic phenomena of cAMP compartmentation in the fine processes and kinase translocation into the nucleus were reproduced in sensory-motor neuron cocultures and intact acutely excised ganglia, showing that they were not artifacts of culturing isolated neurons. Electrophysiological recording in intact ganglia verified that the injected probe did not perturb the cells' excitability or response to serotonin.

Applications of Photochemistry to Probe Signal Transduction

Dr. Lewis Makings studied the mechanism of photodestruction of indo-1, an important fluorescent Ca^{2+} indicator particularly suited to confocal microscopy. He found that indo-1 does not simply bleach to nonfluorescent products but also undergoes photoconversion to a form that is still quite fluorescent but has lost its Ca^{2+} sensitivity. Both processes require oxygen and can be largely inhibited by reducing agents such as ascorbic acid or hydroquinone. Reversibility by reductants argues that the key step in photodestruction is formation of the indo-1 free radical rather than direct oxygen addition to the indole 2,3–double bond as previously thought. A further implication is that covalent at-

tachment of a sacrificial reductant may be useful, especially if the reductant can be regenerated by the reducing intracellular milieu.

The recent upsurge of interest in the labile and reactive molecule nitric oxide (NO) as a putative paracrine neurotransmitter and mediator of excitotoxicity provided encouragement to construct a "caged" form of NO, i.e., a complex form that can be suddenly released by photolysis. Such a molecule would permit NO to be delivered with precise localization and timing, which is not possible now. Preliminary results of Dr. Makings suggest that hemoglobin (Hb) in which Fe(II) has been replaced by Mn(II) may cage NO. $Hb \cdot MnNO$ is isoelectronic with $Hb \cdot FeCO$, which (unlike $Hb \cdot FeNO$) photodissociates efficiently. Further efforts are aimed at optimizing the preparation and stability of $Hb \cdot MnNO$, finding lower-molecular-weight analogues, and applying them to relevant biology.

Chelators that cage Ca^{2+} have been of considerable value in quantifying its triggering role in many important processes, such as muscle contraction and exocytosis. Dr. Stephen Adams serendipitously discovered a new photolabile chelator that is several hundredfold more sensitive to light and undergoes 10-fold larger changes in Ca^{2+} affinity than the best previous molecules, while retaining the $Ca^{2+}:Mg^{2+}$ selectivity that is important for intracellular applications.

Roles of Controversial and Novel Second Messengers

Dr. Carsten Schultz continued exploring membrane-permeant acetoxymethyl (AM) esters of phosphate-containing messengers. He discovered that the dibutyryl AM ester of the controversial messenger inositol 1,3,4,5-tetrakisphosphate (IP_4) sometimes causes depression of elevated cytosolic Ca^{2+} levels back toward basal levels in REF-52 fibroblasts. This result seems more in accord with A. L. Boynton's proposal that IP_4 stimulates reuptake of Ca^{2+} into intracellular stores than with R. F. Irvine's hypothesis that IP_4 opens Ca^{2+} channels in the plasma membrane.

Dr. Schultz also collaborated with Dr. Steven Pandol to show that the permeant IP_4 derivative neither stimulates amylase secretion from pancreatic acinar cells nor adds to the substantial secretion due to the analogous permeant IP_3 derivative. Another collaboration with Drs. Kim Barrett and Alexis Traynor-Kaplan suggested that IP_4 may uncouple Cl^- secretion from Ca^{2+} elevation in T_{84} colon carcinoma cells. This would be a novel physiological action of an inositol polyphosphate downstream from Ca^{2+}.

Dr. Clotilde Randriamampita sought the mysterious messenger that is suspected to be generated by emptying of intracellular Ca^{2+} stores and to signal the plasma membrane to open Ca^{2+} channels to replenish them. If the message can get from the lumen of the endoplasmic reticulum to the plasma membrane, it crosses at least one membrane, so it might be able to cross several and diffuse extracellularly. Dr. Randriamampita has found that activated Jurkat lymphocytes release a diffusible factor that can cause Ca^{2+} influx into nearby macrophages or astrocytoma cells. Collection and analysis of Jurkat supernatants suggest that the factor is heat-stable, trypsin-insensitive, low molecular weight, anionic, and weakly adherent to reverse-phase silica, but does not seem to be any of the obvious candidates such as ATP, ADP, AMP, adenosine, GTP, cGMP, leukotrienes C_4 or B_4, platelet-activating factor, arachidonic acid, or NO. Analysis of this possibly novel autocrine/paracrine messenger is continuing.

Dr. Tsien is also Professor of Pharmacology in the School of Medicine of the University of California, San Diego, and Professor of Chemistry at the University of California, San Diego.

Article

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VISUAL TRANSDUCTION IN RETINA

King-Wai Yau, Ph.D., *Investigator*

The main focus of Dr. Yau's laboratory continues to be on the mechanism of visual transduction in retinal photoreceptors. Three projects were carried out over the past year: 1) measurement of dark rod phosphodiesterase activity in rod outer segments, 2) identification of a new cGMP-gated cation channel subunit in rod cells, and 3) immunocytochemical study of transducin subunits in cones.

Rod Phosphodiesterase Activity in Darkness

In darkness there is a steady influx of cations into the outer segment of rod and cone photoreceptors through a cGMP-activated conductance. Isomerization of the visual pigment by light activates, via a G protein called transducin, a cGMP phosphodiesterase that hydrolyzes cGMP, thus leading to the closure of the cGMP-gated conductance. This stops the cation influx and produces a membrane hyperpolarization as the neural response to light. In darkness there is also a basal level of cGMP turnover due to dark cyclase and phosphodiesterase activities. This basal cGMP turnover can be an important factor in determining a cell's sensitivity to light as well as the kinetics of the light response; essentially, a high dark turnover rate can translate into a low sensitivity to light and fast response kinetics. Thus a difference in the dark cGMP turnover rate may underlie some of the differences in sensitivity and response kinetics known to exist between rods and cones. Dr. Yau's group is comparing the dark phosphodiesterase activities in the two kinds of receptors. So far they have measured the activity in rods, by examining the ability of exogenous cGMP in darkness to activate the cation conductance in a rod outer segment (that has been truncated at the base to allow entry of externally applied cGMP) in the presence and the absence of the drug IBMX, which inhibits phosphodiesterase activity. The difference between the two situations should thus reflect dark phosphodiesterase activity. This comes to a rate of $\sim 1-2/s$ in rods. The dark rate in cones remains to be measured.

New cGMP-gated Cation Channel Subunit in Rods

The cGMP-gated channel, which is unusual among ion channels for its choice of ligand, was first purified and cloned by others from bovine eye. It is a 78-kDa protein with putative transmembrane domains and a characteristic cyclic nucleotide–binding site. Immunocytochemical studies have fur-

ther confirmed its presence in the rod outer segment, and expression in *Xenopus* oocytes has verified its function as a cation channel that is gated by cGMP. Thus it appears that the native channel may be a homo-oligomeric complex. Recently Dr. Yau's group has cloned a protein from human retina that shows $\sim 90\%$ homology to the bovine channel. When expressed in a kidney cell line, this protein also shows cGMP-gated channel activity, suggesting that it is probably the human homologue of the rod channel. Dr. Yau's group then screened a human retinal cDNA library with lower stringency for related proteins. The aim was to clone the homologous channel in cone cells, which from previous work is known to be functionally distinct from the rod channel. A distinct clone was obtained that showed considerable homology to the rod channel clone. The surprise was that when expressed by itself in a cell line, this new clone did not form a functional cation channel. On the other hand, when coexpressed with the rod channel clone, channel activity could be observed that more closely resembled the native channel activity (recorded from amphibian rods) than with the rod clone alone; namely, the flickering openings and closings that are characteristic of the native channel are reproduced by the expressed channel in the presence of this new clone. Evidence from immunocytochemistry using an antipeptide antibody has further verified that the protein encoded by this new clone indeed resides in the outer segments of rods rather than cones in the human retina. Thus it appears that the native cGMP-gated channel in rods is a hetero-oligomeric complex. In retrospect, this is not too surprising, because practically all other ligand-gated ion channels studied so far appear to be made up of more than one subunit species. The hunt for the true cone channel subunit(s) is still in progress.

Cell Biological Studies of the Cone Transducin Subunits

G proteins that mediate signal transduction pathways, such as the phototransduction cascade studied by Dr. Yau's group, are heterotrimers composed of α, β, and γ subunits. The functional specificity of a particular G protein has long been thought to reside in the α subunit, with the β and γ subunits (particularly the β subunit) supposed to play a more or less supporting role. Rods and cones have been known to have different G protein (transducin) α subunits, but the makeup of their other subunits re-

mains unclear. To examine this question, Dr. Yau's group has used peptide antibodies against various known G protein β and γ subunits to stain the retina. The rod and cone cells show different immunostainings for β and γ subunits; essentially, rod outer segments appear to have β_1 and γ_1 subunits, while cone outer segments appear to have β_3 and γ_2 subunits. These findings suggest that β and γ subunits probably also contribute to the functional characteristics of transducin, and may partially account for the different light response properties of rods and cones.

Part of the above work was supported by a grant from the National Eye Institute, National Institutes of Health.

Dr. Yau is also Professor of Neuroscience at the Johns Hopkins University School of Medicine.

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REGULATION OF CELL ACTIVITY BY TRANSMEMBRANE SIGNALS

Edward B. Ziff, Ph.D., *Investigator*

Dr. Ziff's laboratory studies the molecular mechanisms by which growth factors and other transmembrane signaling agents control cellular proliferation and differentiation. They have shown that growth factors rapidly induce the transcriptional activity of specific genes, including the c-*fos* gene, which encodes the transcription factor c-Fos, the prototype of the family of early-response proteins. The c-Fos protein forms a heterodimeric complex with c-Jun protein, which binds to specific DNA sequences and activates transcription of neighboring genes.

For these studies, Dr. Ziff's laboratory employs the rat pheochromocytoma cell line PC12, which responds to the polypeptide hormone nerve growth factor (NGF) by differentiating from a chromaffin-like to a neuronal phenotype. They find that NGF rapidly induces c-*fos* in PC12. The mechanism of induction is being investigated in studies supported by the American Cancer Society. The fact that c-Fos protein is a transcription factor and is controlled by NGF suggests it has a role in regulating gene expression and neuronal differentiation in response to NGF.

Following the rapid induction of c-*fos* by NGF, other genes are induced with slower kinetics. One is the tyrosine hydroxylase (TH) gene, a delayed-early gene induced 1–2 h after NGF treatment that encodes an enzyme that catalyzes the rate-limiting step of catecholamine neurotransmitter biosynthesis. Dr.

Ziff's laboratory has identified a TH promoter regulatory sequence, the TH-FSE, which binds the Fos-Jun complex and is required for NGF induction of transcription of the TH gene. This indicates that NGF induction of c-Fos regulates transcription of the TH gene, thereby providing a mechanism for NGF control of catecholamine biosynthesis. Other stimuli that activate c-Fos, such as Ca^{2+} ion influx following depolarization of neurons, may also regulate TH gene expression through this pathway. The c-Fos protein belongs to a family of proteins, including FosB, Fra1, and Fra2, all of which may bind to Jun or to members of the Jun protein family. The resulting complexes in turn may all bind to the same DNA element. The different c-Fos family members differ in their patterns of expression following cell stimulation, as well as in their structures outside the DNA-binding domains, suggesting they may regulate gene activity differentially when complexed with a DNA element such as the TH-FSE. Recent studies in the Ziff laboratory indicate that the TH gene is repressed by a mechanism in which the c-Fos protein, an activator, is replaced by a different c-Fos family member, which serves as a repressor. Indeed, other Fos family members, including FosB, become the predominant species as c-Fos levels dwindle and TH transcription is shut off.

Another gene induced with a late time course, the peripherin gene, encodes a neuronal-specific type

III intermediate filament protein. The peripherin gene is active at 24 h following NGF treatment, a time when the morphology of PC12 cells begins to change and neurites are extended. Results from the Ziff laboratory indicate that a peripherin promoter negative regulatory element (NRE) releases a protein upon PC12 differentiation, and suggest that dissociation of this potential repressor, coupled with positive signals from other elements, activates the gene. In developing rat embryos, initiation of peripherin protein synthesis coincides with the morphological differentiation of neurons and the transit of the cells to the postmitotic state. Thus, in development, peripherin induction is one constituent of a program of gene expression activated at the terminal stages of neuronal differentiation.

In the terminal differentiation of neurons and other cell types, down-regulation of expression of the c-*myc* gene appears to be a necessary step. In studies supported by the National Cancer Institute, National Institutes of Health, the Ziff laboratory has investigated the c-Myc protein, a nuclear phosphoprotein that contains structural features characteristic of a sequence-specific DNA-binding protein, including helix-loop-helix (HLH), leucine zipper (LZ), and basic region (BR) motifs. These structural motifs are related to the structures that enable c-Fos to bind to Jun and the resulting heterodimer to bind to DNA.

In a search for a protein partner of Myc that could heterodimerize with Myc and enable it to bind to DNA, the laboratory cloned a mouse protein, Myn, which was recognized to be the murine homologue of the human protein Max (cloned by Elizabeth Blackwood and Dr. Robert Eisenman). Max and its murine homologue Myn are virtually identical ~18-kDa polypeptides that contain an HLH-LZ-BR DNA-binding motif similar to that of the Myc proteins and that interact with c-Myc to form a specific DNA-binding complex. Myc protein on its own will not form the dimer complex requisite for binding to DNA. However, Myc and Max dimerize to form a complex that will bind to DNA. Max forms a homodimer that binds to DNA with the same sequence specificity as the Myc-Max heterodimer. These results suggest that Myc-Max and Max-Max dimers compete for the same binding sites in DNA but after binding may exert different regulatory effects on the neighboring DNA regions. Present evidence suggests that these dimeric complexes are activators and repressors of transcription; however, their specific roles are not yet established.

DNA tumor viruses express genes that mimic some of the actions of growth factors and nuclear oncoproteins such as c-Fos and c-Myc. The adenovirus-5 E1a gene is one such gene and encodes a cell-immortalizing protein that binds cellular factors, including the product of the retinoblastoma susceptibility locus, Rb. Rb is a tumor-suppressor protein that exerts a negative regulatory effect on cell growth. E1a stimulates cellular and viral DNA synthesis, proliferation, and transcription of adenoviral and cellular genes. The laboratory finds that expression in PC12 cells of adenovirus-5 E1a proteins results in a loss of chromaffin cell and neuronal markers and causes profound changes in cell morphology. TH, peripherin, the low-affinity NGF receptor, the *trk* protein, the EGF receptor, and other neuronal and chromaffin markers are repressed, while more ubiquitously expressed genes are unaltered. This suggests that these differentiation-specific functions are sensitive to the mechanisms that control cell proliferation and that are perturbed by E1a. The region of E1a binding Rb and a 300-kDa cellular protein is required for these changes. The laboratory has found that this same region is crucial for activating viral early genes, including those encoding DNA replication functions. The laboratory is currently attempting to interrelate pathways used by E1a, growth factors, and early-response proteins to control PC12 cell proliferative and differentiation functions.

Dr. Ziff is also Professor of Biochemistry at New York University Medical Center.

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MOLECULAR GENETICS OF VISUAL SYSTEM DEVELOPMENT IN *DROSOPHILA*

S. Lawrence Zipursky, Ph.D., *Associate Investigator*

Dr. Zipursky's laboratory studies mechanisms of neuronal development in the *Drosophila* visual system. These studies are largely directed toward understanding the molecular and cellular mechanisms utilized to determine cell fates and to establish precise patterns of synaptic connectivity. This system has proved amenable to a multidisciplinary approach using genetic, biochemical, and histological analyses.

Development of the *Drosophila* Visual System

The *Drosophila* visual system comprises the compound eye, which receives light, and the optic ganglia, which process visual information. The eye and the optic ganglia are embryologically distinct. Each is derived from a set of cells that are set aside separately as invaginations of the embryonic ectoderm. The cells within these two primordia proliferate during early stages of larval development, with pattern formation and neuronal differentiation commencing during later larval stages. Dr. Zipursky and his colleagues have been studying three aspects of visual system development: 1) an inductive interaction between two cells in the developing retina, 2) the determination of the optic lobe primordium and the regulation of cellular proliferation within it, and 3) the establishment of the precise pattern of synaptic connections made by photoreceptor neurons with their targets in the optic ganglia.

Cell-Cell Interaction Regulates R7 Development

Dr. Zipursky's laboratory has been studying an inductive interaction between the R8 photoreceptor neuron and a neighboring cell, the R7 precursor cell, in the developing compound eye. The compound eye comprises an array of some 800 identical units called ommatidia, each containing eight photoreceptor neurons (R1–R8 cells) and additional accessory nonneuronal cells. Lineage analysis has clearly established that interactions between cells play a critical role in regulating cell fate determination.

Dr. Zipursky and his colleagues have studied the interaction between the protein products of the *seveneless* (*sev*) and *bride of seveneless* (*boss*) genes in controlling the development of the R7 neuron. Mutations in *sev* and *boss* lead to the R7 precursor cell assuming a nonneuronal fate. The *sev* gene encodes a receptor tyrosine kinase and is required in the R7 precursor cell. In contrast, the *boss* gene, which also encodes a membrane protein, is expressed solely in the R8 cell.

Several lines of evidence indicate that boss is the ligand for the sev receptor. First, boss-expressing tissue culture cells bind specifically to sev-expressing cells. Second, membranes from boss-expressing cells stimulate the sev tyrosine kinase. Third, boss is internalized into the developing R7 photoreceptor cell in a sev-dependent mechanism. Finally, ectopic expression of boss leads to the induction of R7 development in other sev-expressing cells. (These studies were supported by a grant from the National Institutes of Health.)

An intriguing developmental issue is the mechanism by which only one sev-receptor–expressing cell responds to the boss inductive cue during normal development. Through a series of experiments in which R7 development was assessed in different mutant backgrounds, and as a consequence of ectopic expression of boss, it was shown that multiple mechanisms contribute to the specificity of induction. During normal development the sev-expressing cells that contact R8 fail to assume an R7 cell fate as a result of their commitment to alternative fates. Cells located one cell diameter away from R8 are competent to respond to the inductive cue, but fail to do so because boss is tethered to the surface of the R8 cell.

Signal Transduction Downstream from the sev Receptor Tyrosine Kinase

Dr. Gerald Rubin and his colleagues (HHMI, University of California, Berkeley) have shown that the sev signal is mediated by a *Drosophila* homologue of ras. Several laboratories have shown in vertebrate systems that ras regulates the activity of the extracellular-regulated kinases 1 and 2 (ERK1 and ERK2). Dr. Zipursky's laboratory has identified a *Drosophila* homologue of ERK, designated ERK A, which is 80% identical to its vertebrate counterparts and has similar biochemical characteristics. Studies using transgenic flies and classical genetic analysis are in progress to determine whether the *Drosophila* ERK A gene plays a role in signaling in the sev pathway.

Control of Optic Ganglion Development

Dr. Zipursky and his colleagues have been studying two genes that are required for early stages of optic ganglion development. The *sine oculis* (*so*) and *anachronism* (*ana*) genes play critical roles in

the determination and temporal control of optic lobe development, respectively. The *so* gene encodes a homeobox-containing protein whose loss of function results in the inability of the optic lobe primordium on the surface of the embryonic ectoderm to invaginate. The transient and highly specific pattern of expression of *so* in the optic lobe placode prior to invagination is consistent with an early determinative role in this process. The *so* gene also plays a critical and very early role in the developing retina.

The *ana* gene, a heterochronic mutation, plays a novel role in regulating brain development. Loss of *ana* function results in an accelerated development of the optic lobes; neuronal differentiation occurs precociously. This loss of coordination between developmental events in the eye and optic lobes results in a severe defect in the projection patterns of the R cells. The *ana* gene encodes a 70-kDa glycoprotein that is secreted by glial-like cells surrounding the primordium. A model that is currently being tested is the hypothesis that *ana* controls timing in the developing visual system by regulating the rate of cellular proliferation in the optic lobes. (The work on the *ana* gene was supported by a grant from the National Institutes of Health.)

Development of Specific Patterns of Neuronal Connectivity in the *Drosophila* Visual System

The R cells form remarkably precise patterns of synaptic connections in the optic lobes. The eight R cells from each ommatidium send axons, arranged in a precise fashion within a fascicle, into the developing lobes. The R1–R6 cells terminate in the first optic ganglion, the lamina; the R7 and R8 cells project through the lamina and into the medulla, where they make synapses. These axons all project into the brain in a retinotopic manner. Dr. Zipursky and his colleagues are using a variety of genetic screens to identify mutations that affect the development of these projections, as a first step to understanding the underlying molecular mechanisms regulating the formation of specific synaptic connections in the fly visual system.

Dr. Zipursky is also Associate Professor of Biological Chemistry at the University of California School of Medicine, Los Angeles, and Member of the Molecular Biology Institute at UCLA.

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MOLECULAR GENETICS OF SENSORY TRANSDUCTION IN *DROSOPHILA*

CHARLES S. ZUKER, PH.D., *Associate Investigator*

The aim of Dr. Zuker's research program is to elucidate mechanisms used for signal transduction in the visual system. The results obtained from these studies should increase understanding of the molecular basis of sensory reception and information processing and will be useful in understanding abnormalities in the human visual and nervous systems.

Activation of the Visual Cascade

Dr. Zuker's laboratory has been characterizing a number of molecules involved in the activation of the phototransduction cascade. These include the visual pigment molecule rhodopsin, the G protein that couples to the effector, and phospholipase C.

Rhodopsin is composed of a protein, opsin, covalently linked to 11-*cis* retinal. The spectral sensitivity of human rhodopsin and color opsins appears to be regulated by interactions between the 11-*cis* retinal chromophore and charged or polar amino acids within the opsin apoprotein. In an attempt to elucidate the molecular basis of spectral specificity of rhodopsin *in vivo*, a large collection of chimeric visual pigments made up of parts of the blue-sensitive R1–6 opsin and parts of the violet-sensitive R8 opsin were constructed and then reintroduced into flies, where their spectral behavior can be assayed in their normal cellular and organismal environment. Characterization of these transgenic flies showed that it is possible to introduce localized structural changes within the opsin protein that alter its spectral properties. Moreover, it is possible to retune metarhodopsin absorption, the activated form of the molecule, without an associated change in rhodopsin sensitivity. This finding demonstrated that different regions of the protein interact with the chromophore in the native and activated states and opens up the possibility of custom-tailoring light receptor molecules with defined properties.

rdgB (*retinal degeneration B*) is an X-linked mutation that triggers an irreversible course of light-dependent degeneration of the R1–6 photoreceptor cells of *Drosophila*. Activation of the visual cascade is required for the degeneration of the photoreceptors to occur. As a means of identifying novel components involved in the activation of the visual cascade, the laboratory is screening for second site suppressors and enhancers of photoreceptor degeneration in *rdgB*. A number of recessive mutations that protect the photoreceptors of *rdgB* from light-dependent degeneration have been identified. These mutants are being characterized with physiological, genetic, and molecular techniques.

Regulation of the Visual Cascade

Arrestins are a family of proteins thought to mediate the inactivation of G protein–coupled receptors. In photoreceptors, they are thought to uncouple rhodopsin–G protein interaction. Although much is known about arrestin function *in vitro*, little is known about its precise role *in vivo*. Two arrestin genes have been identified in *Drosophila* (*arr1* and *arr2*); both are photoreceptor cell specific. Although it is tempting to speculate that arrestins function in catalyzing the termination of the active state of rhodopsin, the identification of a biochemical activity *in vitro* does not necessarily mimic or underlie a corresponding role for those molecules *in vivo*. Thus the phenotype of mutants defective in arrestin function may not be easily predicted. To circumvent this potential problem, the laboratory successfully completed a screen for arrestin mutants that was based solely on the loss of the protein on Western blots. The physiological and biochemical characterization of these mutants should provide definitive evidence as to the *in vivo* role of arrestins.

To carry out a detailed physiological characterization of the light response of *Drosophila* photoreceptors, Dr. Zuker and his colleagues developed a novel preparation of isolated photoreceptors suitable for patch-clamp analysis. With this preparation, influx of extracellular calcium was shown to be sufficient and necessary for mediating a rapid stimulus-dependent inactivation of the phototransduction cascade. The laboratory has shown that mutations in a gene that encodes a photoreceptor-specific isoform of a protein kinase C (eye-PKC) are specifically defective in this calcium-dependent inactivation mechanism, and the expression of the mutant phenotype requires extracellular calcium. These data suggest a model in which the light-dependent calcium influx rapidly shuts off the light-activated electrical response through activation of eye-PKC. Genetic screens aimed at identifying the upstream regulators and downstream targets of this eye-specific PKC are in progress.

Recently, in collaboration with Dr. Roger Tsien (HHMI, University of California, San Diego), the laboratory has begun to combine patch-clamp recordings of the electrical response with high-speed confocal imaging of the kinetics of calcium mobili-

zation. These experiments should directly reveal the time course of calcium influx and release within the photoreceptor during the light-activated response.

ninaA and Related Cyclophilins

The *ninaA* gene product is a photoreceptor-specific cyclophilin that is required for proper rhodopsin biogenesis. *ninaA* flies have 5–10% of wild-type levels of Rh1 rhodopsin in their R1–6 photoreceptor cells and yet have wild-type levels of the Rh1 mRNA. Cyclophilins are a conserved family of peptidyl-prolyl *cis-trans* isomerases that have been implicated in intracellular protein trafficking and folding and are believed to mediate the effects of the powerful immunosuppressing drug cyclosporin A.

To gain insight into the role of cyclophilins, the laboratory carried out a genetic screen designed to identify functionally important regions in the ninaA protein. More than 700,000 mutagenized chromosomes were screened for a visible *ninaA* phenotype, and 70 independent mutations in *ninaA* were isolated and characterized. This screen, based on a rhodopsin-dependent phenotype and unbiased in its requirement for a defined biochemical activity of ninaA, has provided significant insight into the structure/function relationships important to the natural cellular function of a cyclophilin. Remarkably, most of the changes mapped to or near the β-barrel face of cyclophilin that appears to be involved in cyclosporin A binding and prolyl-isomerase substrate binding. These results provide the strongest argument to date that the functionally relevant region of the molecule, *in vivo*, corresponds to and overlaps with the peptidyl-prolyl substrate-binding site.

In a related set of studies, the gene encoding the *Drosophila* homologue of the abundant, cytosolic form of human cyclophilin was cloned, and several screening strategies are being employed to isolate mutations in this locus. The generation of cyclophilin mutants in *Drosophila* should greatly extend knowledge of the biological role of this class of protein and provide a useful tool for the genetic identification of their intracellular targets.

Mutants Insensitive to the Bat of an Eyelash

Dr. Zuker's laboratory has recently extended its studies into a new aspect of sensory transduction: a search for the molecular basis of mechanotransduc-

tion, the conversion of mechanical stimuli into neuronal signals that underlies the senses of touch, hearing, and balance. The electrophysiology and biophysics of some mechanosensory organs have been described in detail, but, in contrast to the organs of sight, taste, and smell, nothing is known of the molecular identity of their transducers.

Dr. Zuker and his colleagues are identifying genes that encode mechanosensory components in *Drosophila* by isolating mutations that affect touch-induced behavior. In contrast to defects in photoreception, a complete loss of mechanosensation would probably kill an adult fly. Therefore this screen relied on behavioral defects in the larval stages: specifically, a touch-evoked turning and withdrawal of the larva away from a gentle touch with an eyelash. A screen of the X chromosome—20% of the *Drosophila* genome—has yielded 40 mutations with defects in this behavior, ranging from somewhat uncoordinated movement to complete mechanoinsensitivity. A preliminary genetic mapping and sorting shows that at least four genes have been hit multiple times. The mutant lines are now being examined for defects in larval and adult anatomy, behavior, and physiology.

Dr. Zuker is also Associate Professor of Biology and of Neurosciences at the University of California School of Medicine, San Diego.

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STRUCTURAL BIOLOGY

The Institute's Program in Structural Biology is its newest, begun in 1986, with the primary goal of understanding in atomic detail the three-dimensional architecture of proteins, protein assemblies, and the complexes formed by proteins that interact with RNA and DNA. This structural information is being explored to determine how these molecules fold and assume their active configurations and how they interact with each other. Computational tools are also under development to aid in structure determination.

Investigators in the structural biology program are located at Harvard University, the Massachusetts Institute of Technology, Rockefeller University, the University of Texas Southwestern Medical Center at Dallas, the University of Oregon at Eugene, Baylor College of Medicine, Yale University, Columbia University College of Physicians and Surgeons, and the University of California at San Francisco. The Institute is also developing a synchrotron resource for use by its investigators and the larger biomedical community at the National Synchrotron Light Source at Brookhaven National Laboratory on Long Island.

Assistant Investigator Axel T. Brünger, Ph.D. (Yale University) and his colleagues focus their research at the interface between theory and experiment in the area of structural biophysics. A major effort centers on assessing and improving the accuracy of three-dimensional structures of biological macromolecules based on x-ray crystallography and nuclear magnetic resonance spectroscopy. During the past year novel criteria have been developed that show promise in detecting errors and that could prevent researchers from overfitting the experimental data. Another focus of Dr. Brünger's research is the simulation of macromolecular interactions and energetics. Prediction of the geometry of helical supercoils was quite successful in the case of the dimerization region of the GCN4 transcriptional activator protein. The method has also been applied to predict the structure of the glycophorin-A transmembrane domain.

The work of Investigator Wayne A. Hendrickson, Ph.D. (Columbia University) and his colleagues integrates structural studies of significant biological molecules with the development of methods to facilitate these investigations. The major biological themes at present include cell surface interactions, genetic replication, carbohydrate recognition, and oxygen transport. Their research on methods focuses on synchrotron radiation, crystallographic phase evaluation from multiwavelength anomalous diffraction (MAD) measurements, crystallographic refinement, and computational crystallography. During the past year the refined structures of the carbohydrate recognition domain (CRD) from mannose-binding proteins, both unligated and complexed with a high-mannose oligosaccharide, have been used to define the characteristics of sugar binding and selectivity in this system. Other new structures include the T cell co-receptor CD8 and an adhesive type III fibronectin domain from the extracellular matrix protein tenascin.

Structural studies of simple viruses and soluble receptor fragments have revealed important aspects of assembly and recognition. Further progress with more complex structures in the laboratory of Investigator Stephen C. Harrison, Ph.D. (Harvard University) will build on technical advances made during the past year in working on crystals of SV40 (simian virus 40), rotavirus single-shelled particles, and transferrin receptor. New structures of the DNA-binding elements from two yeast transcriptional activators—GAL4 and GCN4—reveal the organization of recognition modules not previously seen at high resolution. The significance of flexible segments is evident in both cases.

The laboratory of Investigator Don C. Wiley, Ph.D. (Harvard University) continues to study the structure and function of cellular and viral surface molecules. In the past year the laboratory determined the structure of a complex between an influenza virus protein and a component of its cellular receptor. This opens the way to designing drugs that would inhibit virus from binding to cells. Using recombinant DNA methods, the laboratory has also studied the membrane fusion mechanism by which viruses enter cells. In other studies the three-dimensional structure was determined for the human histocompatibility antigen, a protein found on all human cells. The function of this protein during the immune response to viral infections and in the rejection of tissue transplantation is now under study.

Investigator Brian W. Matthews, Ph.D., D.Sc. (University of Oregon) and his co-workers use x-ray crystallography, in concert with genetic, thermodynamic, and other techniques, to address some of the fundamental problems in biology: How do proteins spontaneously fold into their biologically active three-dimensional configurations? What determines the stability of these folded proteins? Can stability be improved? How do proteins interact with each other? How do proteins interact with DNA? How do

enzymes interact with their substrates and act as catalysts? Significant progress has been made in using genetic engineering to simplify the protein-folding problem and to quantitate the interactions that stabilize protein structures. The structure of the biotin repressor was determined. Related studies of Cro and other DNA-binding proteins are revealing the structural basis of DNA-protein interaction.

The laboratory of Associate Investigator Robert O. Fox, Ph.D. (Yale University) has developed a cysteine-specific iron chelator reagent that can effect cleavage of peptide bonds. This novel reagent has been useful in mapping the structure of protein-folding intermediates and of protein-DNA complexes, particularly a number of nuclease and myoglobin variants. In a complementary kinetic experiment, progress has been made using nuclear magnetic resonance spectroscopy to identify an early folding intermediate of staphylococcal nuclease.

The work of Assistant Investigator John Kuriyan, Ph.D. (Rockefeller University) and his colleagues is aimed at obtaining atomic-level information about how protein molecules perform their diverse and specific functions. The major tools used are x-ray diffraction and computer simulation. Proteins under investigation include electron transfer enzymes, DNA-binding proteins that are important in the replication of genetic information, and regulatory proteins involved in carcinogenesis. The structures of two new proteins were recently determined. One is a part of the DNA polymerase that replicates the bacterial chromosome during cell division and serves to tether the rest of the replicative machinery onto the DNA. The other protein is known as the SH2 domain and is a key element in the mechanisms used by the cell to respond to external signals.

The general goal of Investigator Thomas A. Steitz, Ph.D. (Yale University) and his colleagues has been to understand the biological function of macromolecules in terms of their detailed molecular structure. The following are among the questions being asked about proteins that interact with nucleic acids: How do the sequence-specific DNA-binding proteins recognize the particular DNA sequence to which they bind? What are the common structural themes among proteins that interact with nucleic acids? How do the template-directed polymerases assure high fidelity in the copying of templates? How does tRNA binding to human immunodeficiency virus (HIV) reverse transcriptase differ from its binding to synthetases? The specific systems being studied for which crystals exist include the catabolite gene activator protein–DNA complexes, *Escherichia coli* lac

repressor, T7 RNA polymerase complexed with T7 lysozyme, the *E. coli* Klenow fragment complex with DNA, HIV reverse transcriptase, T4 gene 32, single-stranded DNA-binding protein–DNA complex, resolvase, recA, ruvC, and GlntRNA synthetase–tRNA^{Gln} complex. In order to understand enzyme mechanisms, site-directed mutagenesis is used to determine the effect of specific mutations on the activity and the three-dimensional structure of the protein and its complexes with substrates. Using the structure of HIV reverse transcriptase complexed with appropriate substrate and inhibitor ligands, new inhibitors will be designed that may serve as anti-AIDS drugs.

Assistant Investigator Stephen K. Burley, M.D., D.Phil. (Rockefeller University) and his colleagues are also interested in developing a detailed understanding of the physical principles that govern the general problem of molecular recognition in biological systems. Many important biochemical processes rely on precise recognition of one macromolecule, usually a protein, by another. The approach of this laboratory is to use x-ray crystallography and other biophysical methods to determine the three-dimensional structures of biological macromolecules and their complexes with DNA, protein, or other ligands. These structures contain a wealth of detail that can be analyzed to provide a functional description of the physical forces that are responsible for mediating recognition.

The laboratory of Investigator Carl O. Pabo, Ph.D. (Massachusetts Institute of Technology) is attempting to understand how proteins recognize specific sites on double-stranded DNA and how the bound proteins regulate gene expression. His group uses x-ray crystallography to determine the three-dimensional structure of protein-DNA complexes. Recent work has revealed how two of the major families of eukaryotic DNA-binding proteins (known as the homeodomain and the zinc finger) recognize their binding sites. This information will eventually be used to help design novel DNA-binding proteins for research, diagnosis, and therapy.

During the past year Investigator Johann Deisenhofer, Ph.D. (University of Texas Southwestern Medical Center at Dallas) and his research group have been working on the analysis of the three-dimensional structure of several proteins by x-ray diffraction methods. The crystal structure of cytochrome P450$_{BM-3}$ was solved, and cytochrome P450$_{TERP}$, DNA photolyase, a fragment of synapsin I, ribonuclease inhibitor, and SecA protein are among other proteins under analysis.

The interaction between a protein and its ligand (large or small) forms the basis of biological speci-

ficity and is in part responsible for the remarkable selectivities exhibited by most enzymes for their substrates, by transport systems for their nutrient, by antibodies for their antigens, and by virus or bacteria for their host. Largely through use of x-ray crystallography, Investigator Florante A. Quiocho, Ph.D. (Baylor College of Medicine) and his colleagues are studying the structures of a number of proteins in order to understand at the atomic level the features associated with a variety of protein-ligand interactions. These include adenosine deaminase, an enzyme required in the normal development of the immune response; calmodulin, a key protein in the activation of target proteins; two antibodies, one raised against a coat protein of the AIDS virus and the other against surface polysaccharide of a bacterium; aldose reductase, an enzyme believed responsible for some diabetic complications; and several periplasmic receptor proteins with specificity for carbohydrates, oxyanions, and amino acids involved in active transport and chemotaxis in bacterial cells.

The laboratory of Associate Investigator Stephen R. Sprang, Ph.D. (University of Texas Southwestern Medical Center at Dallas) has focused on x-ray structural studies of proteins that regulate cellular processes. These include hormones such as tumor necrosis factor and fibroblast growth factor (FGF), both of which are implicated in the induction of cell growth, differentiation, and in the case of FGF, oncogenesis. The group is also interested in the mechanisms by which the signals induced by the interaction of such hormones with cellular receptors are coupled to specific intracellular events. Ongoing structural studies of the α subunit of Gi protein, which couples the muscarinic receptors to activation of potassium channels, are intended to illuminate the molecular basis of signal transduction by members of the G protein family. Dr. Sprang also maintains a long-term research program to study the allosteric mechanism of glycogen phosphorylase.

High-resolution crystallography continues to provide mechanistic insight into transcriptional regulation in the laboratory of Investigator Paul B. Sigler, M.D., Ph.D. (Yale University). Targeting of dimeric steroid/nuclear receptors to their DNA response elements affirms the role of DNA in influencing discrim-inatory protein-protein interactions at the site of control. Studies of E2, the master transcription regulator of papillomavirus, reveal a heretofore undescribed structure—a dimeric β barrel that serves as a scaffold for recognition helices and bends DNA. Crystallographic studies of the rhodopsin–transducin–cGMP phosphodiesterase (PD)–signaling system have progressed to 2.0 Å in the case of the activated $G_{\alpha t} \cdot$ GTP complex. $G_{\alpha t} \cdot$ GTP has also been crystallized in a complex with its target, the γ subunit of PD.

The research of Investigator John W. Sedat, Ph.D. (University of California, San Francisco) and his colleagues centers on the determination of eukaryotic chromosome structure using three-dimensional optical and electron microscopic approaches. This past year, development of multidimensional optical microscopy has made possible the analysis of the spatial and temporal dynamics of topoisomerase II and lamin nuclear proteins. The three-dimensional hybridization *in situ* of selected DNA probes in nuclei within *Drosophila* embryos has been extended in order to investigate homologous chromosome pairing interactions. Work on the architecture and substructure determination of mitotic and meiotic chromosomes using combined optical and electron microscopic analysis is in progress.

The laboratory of Associate Investigator David A. Agard, Ph.D. (University of California, San Francisco) focuses on understanding the relationship between structure and function at the cellular and molecular levels. Recent work using IVEM tomography is providing new insights into higher order chromosome structure. The group has also developed a novel theoretical approach that appears able to predict accurately the functional consequences of a mutation. The unusual folding pathway of the protein α-lytic protease has provided the laboratory with a unique opportunity to trap a stable folding intermediate under nondenaturing conditions and to study the foldase that converts it to the mature, active enzyme. In other studies the crystal structure of apolipoprotein E, an important protein in human cholesterol metabolism, along with several naturally occurring human mutations in this protein, have recently been solved by x-ray crystallography.

UNDERSTANDING THE RELATIONSHIP BETWEEN STRUCTURE AND FUNCTION AT THE MOLECULAR AND CELLULAR LEVELS

DAVID A. AGARD, PH.D., *Associate Investigator*

The research in Dr. Agard's laboratory continues to be devoted to structural studies of biological problems, in an effort to understand the fundamental relationships between structure and function at the molecular and cellular levels. Four areas of investigation are being pursued.

Structural Basis of Enzyme Specificity

One of the fundamental functions of an enzyme is to be specific, that is, to limit the number of substrates on which it can act. Dr. Agard and his co-workers have chosen α-lytic protease as an ideal model system to investigate structural and energetic aspects of enzyme specificity. They are combining x-ray crystallography, site-directed mutagenesis, kinetics, and theoretical approaches in seeking to understand the relationship between enzyme structure and function.

Structural analysis has provided surprising insights into the mechanism of specificity and has indicated that flexibility plays a crucial role in selectivity. Current efforts involve mapping the energetics of protein flexibility through further mutagenesis and kinetic and crystallographic analyses. Recent experiments have shown that specificity can be altered by mutating residues that regulate flexibility. This is the first demonstration of a mechanism whereby substrate specificity can be modulated by residues distant from the binding pocket.

A key test of one's understanding is to be able to predict the effect of mutations on substrate specificity. The Agard laboratory has brought this objective dramatically closer through a newly developed algorithm that combines the side-chain rotamer concept developed by Ponder and Richards with a complete force-field and solvent model. It has been possible to predict 40 experimental values of k_{cat}/K_m for different mutant-substrate combinations for α-lytic protease and 100 values for subtilisin with exceptional accuracy: an average error of <0.7 kcal/mol, or a factor of $\sim$3 in k_{cat}/K_m. This approach has permitted *de novo* design of an enzyme with a new pattern of specificity and has proved very effective for modeling the structure of an unknown protein based on a homologous structure. Current efforts center on the use of this free-energy function to refine the conformation of model-built substrates. This is a key step toward practical, rational drug design.

Pro Regions as a New Class of Molecular Chaperones

α-Lytic protease is synthesized as a preproenzyme. Experiments in the Agard laboratory have demonstrated that the 166–amino acid pro region is absolutely required for the proper folding of the 198–amino acid protease domain, either *in vivo* or *in vitro*. Significantly, the covalent linkage between the pro region and the protease domain is not required for function. In principle, the pro region could function by reducing the rate of off-pathway folding reactions (as suggested for the "classical" molecular chaperonins) or by increasing the rate of a limiting on-pathway reaction. The laboratory has recently found that the pro region is a potent inhibitor of the mature protease. This suggests that the pro region directly facilitates an on-pathway reaction and that the rate-limiting folding transition state has a native-like conformation.

Remarkably, the Agard group has been able to trap a stable folding intermediate under nondenaturing conditions at physiological pH. This provides a unique opportunity to examine the folding processes in detail, structurally and functionally. The intermediate rapidly folds to the native state upon addition of the pro region. Detailed kinetic analysis indicates that the pro region promotes folding by directly speeding the rate-limiting step on the folding pathway by >10^7. By mutagenesis, it appears that a residue in the pro region that plays a "catalytic" role has been isolated. This is a major step toward understanding the mechanism of folding catalysis. Recent work has also revealed that the intermediate has many features of a molten globule: expanded radius, considerable secondary structure, and little or no tertiary structure. Current work focuses on further structural and functional characterization of the folding intermediate and of the pro region complexed with native protease, using genetics, spectroscopy, nuclear magnetic resonance (NMR), and x-ray crystallography. This study has also provided new insights into the mechanism of secretion of proteins through the outer membrane of *Escherichia coli*.

Three-Dimensional Analysis of Chromosome Structure

The Agard laboratory is engaged in a close collaboration with Dr. John Sedat (HHMI, University of Cali-

fornia, San Francisco) in all aspects of the investigations of chromosome structure. The primary aim of this collaborative effort is to provide a physical basis for understanding chromosome behavior and function through direct determination of the three-dimensional structure of eukaryotic chromosomes as a function of both transcriptional state and cell cycle stage.

Chromosomes are extremely complex, dynamic structures that have defied definitive analysis for over 100 years. The general approach has been to disrupt the structure, then to try to infer what the intact structure was like. Not surprisingly, this approach has been largely unsuccessful. The Agard and Sedat laboratories, however, have concentrated on applying high-resolution electron microscope (EM) tomography to overcome the extraordinary complexity of intact chromosomes. From these studies, it has been learned that both the radial-loop and sequential helical coiling models of chromosome structure are gross oversimplifications. In reality, mitotic chromosomes are built from a fundamental nucleosomal fiber of $\sim$110 Å in diameter, which is organized into higher-order structures measuring 1,300 Å in diameter.

During the past year the group has continued to concentrate on the structure of this 1,300-Å fiber. Although much work remains, current data support a new model in which the nucleosomal fiber is folded back and forth upon itself to form a local domain that is then supertwisted about the 1,300-Å fiber axis. Efforts have been focused on improving sample preservation, staining methods, and resolution of the three-dimensional reconstructions. Experiments in the use of high-pressure freezing, cryo-embedding, and a new DNA-specific stain are now under way.

A key breakthrough in the past year has been the development of fully automated EM tomographic data collection. Not only does this greatly simplify the arduous task of collecting the 120 tilted views required for a reconstruction, but it limits the electron beam exposure by 100-fold. When coupled with cryotechniques, this approach should effectively eliminate beam damage. The quality of the reconstructions should be greatly improved and beam-sensitive materials opened to tomographic study. Furthermore, the laboratory is making great progress in automating the rest of the reconstruction process, paving the way for this powerful technique to be useful to the entire cell biology community. (The project described above was supported in part by a grant from the National Institutes of Health.)

Structure of Apolipoprotein E

Apolipoprotein E (apo-E) is an important protein in human cholesterol metabolism. In specifically binding to the low-density lipoprotein (LDL) receptor, apo-E mediates cellular uptake of high-density lipoprotein (HDL), very low density lipoprotein (VLDL), and chylomicrons. Previously the Agard group had solved the structure of the 22-kDa receptor-binding domain of apo-E to 2.25 Å. This work reveals that the protein is organized as an unusually long four-helix bundle. Although the surface is highly polar, most of the charged groups participate in salt bridges.

Recently the Agard laboratory solved the structure of the two most common human mutant forms of this domain. One of these was known to knock out receptor binding, yet was not located in the putative receptor-binding region. Structural analysis indicates that this mutation causes a dramatic reorganization of the salt bridges, so as to recruit a key receptor-binding residue into a new salt bridge. This represents a novel mechanism for action at a distance. Current work focuses on producing soluble fragments of the LDL receptor to permit structural analysis of complexes with apo-E.

Dr. Agard is also Associate Professor of Biochemistry and Pharmaceutical Chemistry at the University of California, San Francisco.

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COMPUTER SIMULATION OF BIOLOGICAL MACROMOLECULES

Axel T. Brünger, Ph.D., *Assistant Investigator*

Dr. Brünger's research focuses at the interface between theory and experiment in structural biophysics. The current efforts center on studies of macromolecular structure, interaction and energetics, and methodological developments in x-ray crystallography and nuclear magnetic resonance (NMR) spectroscopy. The research tools are a variety of simulation methods for macromolecules, including molecular dynamics, conformational searching, and simulated annealing.

Crystallography: Assessment of Atomic Models and Phases

In the past decade macromolecular crystallography has undergone major advances in crystallization, data collection by synchrotron x-ray sources and area detectors, and data analysis by high-performance computers and new computational techniques. In addition, recombinant gene technology often allows the expression of large amounts of protein. This has resulted in an unprecedented increase in the number of protein structures elucidated.

Despite these successes, the fundamental problem in x-ray crystallography, the phase problem, remains unchanged. That is, from a monochromatic diffraction of a single crystal, it is possible to obtain the amplitudes but not the phases of the reflections, whereas construction of the electron density by Fourier transformation requires both components of the complex structure factors. Phase information has to be obtained through experimental approaches, most commonly multiple isomorphous replacement or knowledge-based procedures referred to as Patterson search or molecular replacement. Phase information obtained through these techniques is usually of limited accuracy and resolution, making it difficult sometimes to interpret elec-

tron density maps in certain regions of the molecule. Furthermore, macromolecular crystals usually diffract to less than atomic resolution, compromising the process of fitting an atomic model to the observed intensities.

Clearly, it is imperative to obtain crystal structures with maximum correctness and accuracy. This applies to their interpretation in terms of macromolecular function and interaction, to their incorporation into databases that are the foundation for structure prediction and other theoretical studies, and to their use in designing drugs on the basis of structural information.

Cross-validation, a tool of modern statistics, can be used to assess the quality of a model that is fitted against observed data. The idea behind cross-validation is deceptively simple: it consists of omitting a certain fraction of the data while the atomic model is fitted to the remaining data. The agreement between the model and the omitted data serves as an unbiased measure of the quality of the fit. Dr. Brünger realized that cross-validation could be applied to the traditional measure of the fit between an atomic model and the diffraction data: the R value. Despite stereochemical restraints, it is possible to overfit, or misfit, the diffraction data: an incorrect model can be refined to fairly good conventional R values, as several publications of (partially) incorrect crystal structures have shown. The "free" R value is defined as the agreement between observed and computed structure-factor amplitudes for the omitted test reflections.

Examples showed that the free R value is a much better measure than the conventional R value for distinguishing between correct and incorrect atomic models. Furthermore, the free R value can be used to assess the improvement in the fit of an atomic model to the observed diffraction data by

introducing additional parameters or chemical restraints. Perhaps the most striking result is a high correlation between the free R value and the model's phase accuracy. In other words, the accuracy of the model's phases can be assessed with the observed amplitudes.

This suggests applications of the free R approach in the area of *ab initio* phasing. An example is the modeling of diffraction data by a liquid of atomic point scatterers, which shows some promise for either very high or very low resolution phasing. Because of the simplicity of the liquid, configurational space can be searched efficiently. However, at medium resolution ranges typically obtainable for macromolecules, there are a large number of configurations with R values that are equally as good as those of the correct solution. There appear to be fewer configurations with free R values equally as good as those of the correct solution. It is thus conceivable that the free R approach could extend the applicability of the liquid model.

The project described above was supported in part by a grant from the National Science Foundation.

NMR Spectroscopy: Accuracy and Precision of Solution Structures

Parallel to the development of criteria for the accuracy of crystal structures, Dr. Brünger's group has embarked on similar investigations for solution NMR-derived structures. The theory that describes NMR relaxation processes based on atomic models is less well developed than in x-ray crystallography, where the atomic model is essentially related to the diffraction data by a Fourier transformation. In contrast, NMR relaxation processes depend on atomic motions over a wide range of time scales, which are difficult to simulate with current methodologies and computing power. Furthermore, solution NMR-derived structures are usually less well determined than x-ray crystal structures; the observed ^1H nuclear Overhauser enhancement (NOE) intensities show large errors; and efforts to improve the fit of the model to the NOE intensity data are prone to overfitting.

The intensity of the observed NOE cross-peaks is a function of certain interproton distances, thus providing information on the three-dimensional structure of the studied molecule. For a rigid spin system and short mixing times in a first approximation, the NOE intensity between a pair of protons is directly proportional to the inverse of the sixth power of the distance separating the protons (isolated spin-pair approximation). In the case of large molecules, the relationship between the cross-peak intensity and the distance between the two protons is more complicated, since indirect magnetization transfer via other protons ("spin diffusion") contributes as well. This, together with uncertainties in the motional behavior of a biomolecule, allows only within approximate ranges the determination of interproton distances derived with the isolated spin-pair approximation. Until very recently structure determination with NMR data has relied on these approximate distance ranges.

Dr. Brünger's group has begun to apply complete matrix relaxation methods to a number of systems that, at least in principle, can circumvent the isolated spin-pair approximation. In this approach the differences between the NOE intensities and those calculated by the full relaxation matrix approach are directly minimized. Chemical restraints are added in a fashion similar to crystallographic refinement.

The results can be summarized as follows. Upon refinement of the macromolecule, the fit to the NMR data improves significantly with relatively small shifts (typically 0.5–1 Å) in the refined structures. For example, in the case of the squash trypsin inhibitor CMTI, the refined structures are somewhat closer to the x-ray structure of the inhibitor (actually, a complex of CMTI with trypsin) than are the initial structures. The deviations between observed and computed NOEs are significantly improved, except for NOEs that are either misassigned or inappropriately modeled as a result of conformational averaging.

Structure Prediction of Helical Supercoils as Applied to Leucine Zippers and Membrane Proteins

While *ab initio* prediction of protein structure is an elusive goal, prediction of tertiary structure from secondary structure using "docking" procedures is more likely to succeed. Although the general principles of protein association seem fairly well understood, the application of these principles to the prediction of specific association remains difficult, even for a seemingly straightforward case. Simple criteria such as buried surface area, solvation free energy, electrostatics, or packing are insufficient to predict correct association, while empirical energy functions are prone to inaccuracies and subject to the multiple minima problem.

Under certain minimal assumptions about helix-helix association, it is possible to guide the docking process. An automated approach for the modeling of helical supercoils through simulated annealing was developed previously by Dr. Brünger's group. Initially, a model consisting only of C^α atoms is cre-

ated, representing an uncoiled helical dimer. Human bias in the placing of the other atoms is reduced by an automatic building procedure that employs simulated annealing with simple geometric restraints. The resulting all-atom model is then allowed to relax during a short molecular dynamics run *in vacuo*, using an empirical energy function and weak restraints that reflect the helical supercoil assumption. Several models can be obtained by using different initial conditions for the simulated annealing procedure. These models can be further refined through use of unrestrained molecular dynamics in the appropriate environment.

As a first step toward understanding the specificity of protein-protein interactions in leucine zippers, Dr. Brünger's group applied the simulated annealing procedure to the dimerization region of the transcriptional activator protein GCN4. The predicted models were obtained prior to the publication of the x-ray structure of GCN4 by Dr. Thomas Alber's group (University of Utah). The predicted models turned out to be fairly close to the x-ray structure.

Both the predicted structure and the x-ray crystal structure are close to a classical left-handed coiled coil conformation. The local helix-helix crossing angle of the x-ray structure falls within the range predicted by the models; a slight unwinding of the coiled coil toward the amino-terminal DNA-binding end of the dimerization region has been correctly predicted. Distance maps between the helices are largely identical. The region around asparagine 20 is asymmetric in the x-ray structure and in the models. Surface side-chain dihedrals showed a large variation in the models as expected, because of the highly solvent-exposed surface area of the leucine zipper.

Recent studies suggest specific roles for transmembrane helix interactions in a range of functions, but understanding of the conformation and energetics of such interactions has been elusive. Dr. Brünger's group has studied the dimerization of the transmembrane helix of glycophorin-A by the simulated annealing procedure and has tested the models against mutational analysis data obtained by Dr. Donald Engelman's group (Yale University).

It was found that the dimer is a right-handed supercoil, that an extensive region of close packing lies along the dimer interface, and that there is good agreement with the mutagenesis data. Furthermore, a sequence-specific propensity for a right-handed supercoil was observed when the simulated annealing modeling was started from a dimer of uncoiled helices. Upon replacement of the simulated glycophorin-A sequence by an 18-residue fragment of the dimerization region of the transcription factor GCN4, the system shows a high propensity for a left-handed supercoil. This result supports the hope of predicting folded transmembrane protein structures in the hydrophobic region of the lipid bilayer.

Thermodynamics of Protein-Peptide Interactions

Internal packing effects are thought to be important in determining protein structure and stability. Packing effects also participate in recognition and binding, and their analysis is a step toward rational methods of drug design. Several authors have studied the effect that substitutions of buried hydrophobic residues have on protein stability. Mutation effects can be very complex, with small changes in stability resulting from large enthalpy-entropy compensation, and from many large, mutually canceling, atomic contributions, involving both protein and solvent and both folded and unfolded states. Theoretical work can help in understanding the details of these microscopic effects. Free-energy calculations are one such approach.

The hydrophobic interactions between the S peptide and S protein in the ribonuclease-S complex were investigated using molecular dynamics simulations and free-energy calculations, in a collaboration with Dr. Frederic Richards (Yale University). Three mutations at the buried position Met13 were simulated—M → L, L → I, and I → V—for which x-ray structures and experimental thermodynamic data were available. The calculations gave theoretical estimates of the changes in binding free energies associated with these mutations. The calculated free-energy differences were small (0–1.6 kcal/mol), in agreement with experiment. However, the simulated structures deviated significantly from the experimental ones, and a large uncertainty in the calculated free energies ($\sim$2 kcal/mol) arose from the multiple minima problem. Indeed, multiple conformations are available to the side chains around the mutation site, and the sampling of dihedral rotamer transitions is limited, despite long simulations.

The uncertainty due to multiple conformations is much greater than that due to random statistical errors. Thus the general criterion for a precise simulation is not merely to reduce the random statistical error, as has been suggested, but rather to sample all the important local minima along the mutation pathway and to reduce the statistical error for each one. The calculations suggest that the packing changes associated with the mutations are energetically small and localized, and largely cancel when the complex and the S peptide are compared.

Dr. Brünger is also Associate Professor of Molecular Biophysics and Biochemistry at Yale University.

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BIOPHYSICAL STUDIES OF EUKARYOTIC GENE REGULATION AND MOLECULAR RECOGNITION

Stephen K. Burley, M.D., D.Phil., *Assistant Investigator*

Dr. Burley's laboratory is studying the problem of molecular recognition in biological systems. The investigations are aimed at developing a detailed understanding of the physical principles that govern molecular recognition. Through x-ray crystallography and other biophysical techniques, Dr. Burley and his colleagues determine and characterize the three-dimensional structures of biological macromolecules and their complexes with DNA, proteins, or other ligands. Thus a wealth of atomic detail is available for analysis, using biochemical, molecular genetic, and theoretical methods to provide a functional description of the intra- and intermolecular interactions responsible for stabilizing macromolecular complexes and thereby mediating molecular recognition.

The proteins, or transcription factors, involved in regulating levels of eukaryotic gene expression provide useful model systems for studying the mechanism(s) by which a protein can recognize a specific region of DNA. In addition, the transcriptional control machinery is a good source of model systems for protein-protein recognition, which also occurs routinely during transcription of genes in eukaryotes. Dr. Burley's laboratory has been combining x-ray crystallographic structure determinations of transcription factor–DNA complexes with biophysical characterization of these protein–nucleic acid assemblies.

Crystal Structure of TATA-Box Binding Protein

In collaboration with Drs. Robert G. Roeder and Nam-Hai Chua (Rockefeller University), Dr. Burley and his co-workers are studying the three-

dimensional structure and function of the TATA-box binding protein (TBP), formerly known as recombinant transcription factor IID. TBP, which is required for transcription of eukaryotic genes by all three RNA polymerases, has been highly conserved in evolution. This universal transcription factor does not act alone in higher organisms. Instead, it functions as part of a large multiprotein assembly, consisting of TBP and various TBP-associated factors (TAFs).

In class II gene transcription, the TBP-TAF complex binds to the TATA consensus sequence and directs accretion of a set of general transcription factors to form the preinitiation complex, which stabilizes interactions between RNA polymerase II and the promoter. The role played by TBP in transcription by the other two RNA polymerases is not as well characterized. However, it is believed that TBP functions with a discrete non-overlapping set of TAFs that are polymerase specific. The immediate challenge in understanding the problem of transcriptional regulation of eukaryotic gene expression is to explain how TBP can have such extreme functional versatility.

Dr. Burley and his co-workers have overexpressed and purified large amounts of TBP from various eukaryotes, including *Homo sapiens, Arabidopsis thaliana, Drosophila melanogaster, Saccharomyces cerevisiae*, and *Schizosaccharomyces pombe*. Native crystals of TBP from *S. cerevisiae* and *A. thaliana* (both isoforms) have been obtained.

During the past year Dimitar Nikolov solved the three-dimensional structure of TBP isoform 2 from *A. thaliana* at 2.58-Å resolution, using the multiple isomorphous replacement method. The structure is a novel, highly symmetric, saddle-shaped α/β protein that sits astride the DNA and presents its convex surface for interaction with TAFs and other transcriptionally active proteins. Nikolov continues his attempts at crystallization and structure determination with the remaining TBPs.

Cocrystals of some of the TBPs complexed with duplex oligonucleotides bearing the TATA consensus sequence have also been obtained, and determination of the structure of the transcription factor–DNA complex is under way. Finally, these crystallographic studies are being complemented with detailed biophysical comparisons of these structurally and functionally related proteins and their complexes with DNA.

Crystal Structure of Human Hepatocyte Nuclear Factor-3

In collaboration with Dr. Eseng Lai (Memorial Sloan-Kettering Cancer Center), Dr. Burley and his co-workers are engaged in structural and functional studies of human hepatocyte nuclear factor-3 (HNF-3). This group of transcriptionally active proteins belongs to a gene family in mammals that is homologous to the *Drosophila* homeotic gene *fork head*. These diverse proteins share a highly conserved DNA-binding region, which is thought to represent an entirely new type of DNA-binding motif. Dr. Burley's group overexpressed and purified the DNA-binding domain of two members of the HNF-3 family.

During the past year Dr. Kirk L. Clark has obtained cocrystals of HNF-3γ with a duplex oligonucleotide bearing the DNA recognition sequence. He has measured x-ray diffraction data at 2.8-Å resolution from native and three heavy-atom derivative crystals and will soon complete the determination of the multiple isomorphous replacement structure. Thereafter, the structure of HNF-3α bound to its DNA recognition sequence can be solved by molecular replacement using previously obtained crystals.

Dr. Burley is also Assistant Professor and Co-Head of the Laboratory of Molecular Biophysics at the Rockefeller University.

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THREE-DIMENSIONAL STRUCTURES OF BIOLOGICAL MACROMOLECULES

Johann Deisenhofer, Ph.D., *Investigator*

Dr. Deisenhofer's laboratory studies the three-dimensional structures of biological macromolecules by the methods of x-ray crystallography. The general aim is to understand folding, structural stability, and function. At the focus of interest are protein-protein interactions, the structure of membrane-spanning and membrane-associated proteins, photochemical energy conversion, energy transfer, and electron transfer reactions.

Cytochrome P450 Enzymes

Cytochrome P450s are heme proteins found in all eukaryotes and in some bacteria. In the presence of O_2, NAD(P)H, and additional electron transfer proteins, they function as monooxygenases and catalyze the oxygenation of hydrocarbon compounds of both endogenous and exogenous origin. In collaboration with Dr. Julian A. Peterson and his colleagues (University of Texas Southwestern Medical Center at Dallas), two P450 enzymes were crystallized: the hemoprotein domain of $P450_{BM-3}$ from *Bacillus megaterium* and $P450_{TERP}$ from *Pseudomonas putida*. The former diffracts x-rays to 1.5-Å resolution and the latter to ~2.4-Å resolution. The amino acid sequence of either protein is less than 25% identical to that of $P450_{CAM}$, the only P450 enzyme of known three-dimensional structure.

The structure of $P450_{BM-3}$ was solved by multiple isomorphous replacement and twofold noncrystallographic symmetry averaging. The current model has an R value of 0.26 at 2.5-Å resolution. The structure analysis of $P450_{TERP}$ is hindered by a crystal unit cell dimension of 458 Å, too large for the data collection facilities in the laboratory. Data from native crystals and from potential heavy-atom derivatives were collected at the Cornell synchrotron and are under analysis.

DNA Photolyase from *Escherichia coli*

DNA photolyase is a DNA repair enzyme that uses light energy to split carbon-carbon bonds between neighboring pyrimidine bases produced, e.g., by ultraviolet irradiation. The enzyme is a complex of a polypeptide chain of 471–amino acid residues and two chromophores: FAD (flavin-adenine dinucleotide) and 5,10-methenyltetrahydrofolate. FAD is absolutely required for enzyme activity, while the folate molecule acts as a "light-harvesting" group that transfers electronic excitation energy to the FAD. Dr. Aziz Sancar (University of North Carolina at Chapel Hill) has provided purified protein.

Crystals of DNA photolyase, with two molecules related by local twofold symmetry in the triclinic unit cell, were obtained. Diffraction data to 2.2-Å resolution were collected from native crystals. One promising candidate for a heavy-atom derivative has been identified, and a search for additional derivatives is under way. In parallel, the possibility of solving the structure by the MAD (multiwavelength anomalous dispersion) phasing method is being explored. (This project is also supported by a grant from the Welch Foundation.)

Synapsin I from Bovine Brain

Synapsin I, a neuron-specific phosphoprotein, is a substrate for cAMP- and Ca^{2+}/calmodulin-dependent protein kinases. It occurs in two closely related isoforms, called synapsin Ia and synapsin Ib, with 706 and 670 amino acids, respectively. The difference between the isoforms is restricted to a region near the carboxyl terminus, beginning at residue 662. Synapsin I interacts with synaptic vesicles and actin filaments and plays an important role in the release of neurotransmitters. Its function appears to be regulated by phosphorylation at three serine residues.

Synapsin I was purified in collaboration with Dr. Thomas Südhof (HHMI, University of Texas Southwestern Medical Center at Dallas). Small crystals that diffract x-rays to ~10-Å resolution turned out to be built from a proteolytic fragment of synapsin I about half the size of the intact protein. Similar fragments expressed in *E. coli* could be crystallized with dramatically improved diffraction quality. Collection of diffraction data to 3-Å resolution and a search for heavy-atom derivatives are in progress.

Ribonuclease Inhibitor from Porcine Liver

Ribonuclease inhibitor (Rnasin) from pig liver is an intracellular protein of 456 amino acids that forms extremely tight 1:1 complexes with ribonuclease A and angiogenin. Its physiological role is as yet unclear. The amino acid sequence of Rnasin consists mainly of seven leucine-rich repeats of 59 residues each. It also contains 30 cysteine residues, all of which appear to be in the reduced state. Similar leucine-rich repeats occur in many other proteins in various numbers, but these proteins are functionally unrelated to Rnasin.

Rnasin was purified from pig liver and crystallized. The tetragonal crystals diffract x-rays to ~3-Å resolution and contain two molecules, related by

local twofold symmetry, per asymmetric unit. To date, diffraction data from native crystals and from seven candidates for heavy-atom derivatives have been collected. One of the derivatives appears to be useful for phasing. Attempts to extend these studies to human Rnasin, and to complexes of porcine and human Rnasin with ribonuclease A, are under way.

SecA Protein from *Escherichia coli*

The SecA protein is a central component of the machinery that performs protein export through the inner membrane of *E. coli*. It consists of a single polypeptide chain of 901 amino acids. Recent findings indicate that it binds signal peptides, has ATPase activity, and can associate with membranes or with integral membrane protein complexes. Dr. Donald B. Oliver and his colleagues (State University of New York at Stony Brook) provided purified SecA protein and, later, raw material for purification.

Well-shaped crystals were grown, but they do not exceed 0.1 mm in any dimension and do not produce a measurable diffraction pattern with a laboratory x-ray source. Investigation of the crystal by electron microscopy revealed that the unit cell is very large, containing probably eight molecules in the asymmetric unit, but the packing is nevertheless very loose.

Currently, the crystallization experiments with various truncated forms of the SecA protein continue.

Other Projects

The subjects of additional projects include the catalytic domain of human HMG-CoA (3-hydroxy-3-methylglutaryl coenzyme A) reductase, UvrA and GroES from *E. coli*, the knob protein from adenovirus, cytochrome b/c$_1$ complexes from *Rhodospirillum rubrum* and beef heart mitochondria, the photosynthetic reaction center from *Rhodobacter capsulatus*, vertebrate phosphofructokinases, and bovine rab3A.

Dr. Deisenhofer is also Regental Professor, Holder of the Virginia and Edward Linthicum Distinguished Chair in Biomolecular Science, and Professor of Biochemistry at the University of Texas Southwestern Medical Center at Dallas.

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BIOPHYSICAL GENETICS OF PROTEIN STRUCTURE AND FOLDING

ROBERT O. FOX, PH.D., *Associate Investigator*

Dr. Fox and his colleagues are investigating the role of the amino acid sequence in determining the folding pathways and the final detailed three-dimensional structure of globular protein molecules. Several experimental approaches are under development to investigate the "structure" of protein molecules in the unfolded and molten globule states. The laboratory also focuses on the role of amino acid sequence in determining turn and loop structures in globular proteins, using staphylococcal nuclease as a model system.

Mapping Structure in the Unfolded and Molten Globule States of Proteins

Many protein molecules fold into defined three-dimensional structures spontaneously and rapidly upon completion of synthesis *in vivo* or upon removal of chemical denaturants *in vitro*. Nonnative states of proteins that are thought to represent steps on the protein-folding pathway can be populated at equilibrium. Under native conditions, fragments of proteins, such as staphylococcal nuclease, display a moderately compact state without applicable sec-

ondary structure, providing an equilibrium model for the early "hydrophobic collapse" phase of protein folding. Under acidic conditions, certain proteins, such as apomyoglobin, enter a molten globule state with some secondary structure but without a defined tertiary structure. The molten globule is thought to represent a later step in the protein-folding pathway. These nonnative states are often difficult to study by current biophysical methods.

To explore the structure of the model protein-folding intermediates described above, a chemical approach has been developed to map close contacts between a variable reporter residue site and all other residues in the protein. A polar iron chelator has been developed that can be specifically attached to cysteine residues engineered into the protein chain. In the presence of ascorbate, the protein chelator adduct generates hydroxyl radicals, which in turn cleave accessible peptide bonds in close proximity to the chelator. The cleavage pattern has been examined for a number of nuclease and apomyoglobin variants. The approach provides structural information for nonnative protein conformations, such as the apomyoglobin molten globule. The cleavage of a small peptide-reagent adduct has been examined in great detail, leading to the proposal of a new cleavage mechanism involving diffusible hydroxyl radicals.

Characterization of Folding Intermediates in Staphylococcal Nuclease

The protection of backbone amide hydrogens from exchange with solvent protons during protein-refolding kinetic experiments has been used to examine the onset of secondary structure formation for several protein systems in a number of other laboratories. In each case, nuclear magnetic resonance (NMR) spectroscopy has been used to follow the extent of protection of many assigned amide protons, providing detailed structural information for folding intermediates.

This year such an experimental approach was applied to staphylococcal nuclease to complement the cleavage-mapping approach described above. In these experiments, amide-deuterated nuclease P117G was rapidly diluted into an H_2O-based refolding buffer at low pH, where the intrinsic amide exchange rate is slow. After a variable folding time (5 ms to several seconds), the protein was "pulsed" in a high-pH buffer in water. Under this condition, backbone amide hydrogens exposed to solvent exchange rapidly, while hydrogen-bonded amides were protected from exchange. The protein finished folding at low pH, where exchange was again slow.

Suitable NMR samples were prepared in a D_2O buffer, two-dimensional proton COSY (correlated spectroscopy) spectra collected, and amide cross-peak volumes integrated to assess the extent of amide protection. The results indicate that a subset of amide protons are partially protected early in the folding pathway, while others are protected only gradually with time. The COSY cross-peaks are currently being assigned for the nuclease P117G variant to the amino acid sequence and structure in order to identify the early-folding domain.

Genetic Analysis of a β-Turn

A genetic system has been developed in the laboratory to explore the amino acid sequence requirements for the formation of a β-turn distant from the active site in staphylococcal nuclease (residues 27–31). A combinatorial library over those positions has been constructed and screened with the aid of a DNase plate assay. A statistical analysis of the resulting sequence data indicates that there are strong and different biases for and against many residues at each of the positions of the type I' β-turn.

Sufficient sequence and activity data have now been collected to allow a linear model for β-turn formation to be developed. The resulting model predicts turn formation in the nuclease experiment and is also successful in predicting type I' β-turns in other proteins of known crystal structure. Crystal structures of three nuclease β-turn variants suggest the type I' β-turn present in the wild-type protein should be preserved in a large majority of the variants examined. A thermodynamic analysis of this library of β-turn variants is in progress. (This work is supported by a grant from the National Institutes of Health.)

Role of Amino Acid Sequence in Determining β-Turn Type

Staphylococcal nuclease occurs in at least two folded conformations that are in slow exchange on the NMR time scale. The interconversion of these folded conformers and the thermal unfolding kinetics have been investigated by magnetization-transfer NMR experiments. Previous work indicates that this conformational heterogeneity is due to *cis-trans* isomerization about the peptide bond preceding proline-117, which is predominantly in the *cis* configuration in the crystal structure. The nuclease conformational equilibrium provides an opportunity to address the physical basis of the relationship between the amino acid sequence and protein conformation. The coexistence of two native conformations in slow exchange on the NMR time scale allows a systematic experimental investigation into

the free-energy contribution of amino acid side chains to the relative stabilization of these native structures.

The goal of this line of investigation is to generate an accurate body of thermodynamic and structural data for this system, using NMR spectroscopy and x-ray crystallography, for use in testing and calibrating molecular energy simulations of proteins. The computational aspect of this project is carried out in collaboration with Dr. Axel Brünger (HHMI, Yale University). This year Dr. Fox and his colleagues have identified several amino acid substitutions that perturb the *cis-trans* equilibrium and have derived a structural hypothesis for these observations based on high-resolution crystal structure determinations. (This work also is supported by a grant from the National Institutes of Health.)

Toward Structural Studies of the Glycine Receptor Channel

Despite intensive biochemical investigations of many voltage- and ligand-gated ion channels, little is known about their molecular structure and mechanisms of gating or ion selectivity. The glycine receptor is an inhibitory chloride ion channel found in the spine, hindbrain, and visual system that is gated by glycine and targeted by strychnine action. While the natural channel is a pentameric assembly of α (48-kDa) and β (58-kDa) subunits associated with a 93-kDa peripheral protein, channels can be formed from the α chain alone that exhibit the conductance and pharmacological properties of the natural channel.

The human glycine receptor $\alpha 1$ chain has been overexpressed in insect cells with a recombinant baculovirus vector. The expression level is high, with little heterogeneity in apparent molecular weight. Whole-cell recordings of infected cells display a large conductance when the cells are exposed to glycine, but not if they have been pretreated with strychnine, indicating that function channels have assembled in the plasma membrane. Purification of sufficient protein for three-dimensional crystallization and crystal structure determination is in progress.

Dr. Fox is also Associate Professor of Molecular Biophysics and Biochemistry at Yale University.

Articles

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STRUCTURAL STUDIES OF VIRUSES, RECEPTORS, AND TRANSCRIPTIONAL CONTROL PROTEINS

Stephen C. Harrison, Ph.D., *Investigator*

Structural studies of macromolecular complexes are aimed at discovering basic molecular mechanisms in cell organization. Dr. Harrison's laboratory has focused on three broad areas for x-ray crystallographic analysis of assembly and recognition: viruses and their interactions with cells, cell-surface receptors, and complexes of transcriptional regulatory proteins with their DNA-binding sites.

Viruses and Viral Proteins

Building on the successful completion of structures for SV40 (simian virus 40) and polyomavirus, using x-ray crystallography, the laboratory has been working on ways to improve the accuracy and reso-lution of these structures and on methods and programs for recording good diffraction data from crystals with even larger unit cells. As a result, data to ~ 3.2-Å resolution have been collected from SV40 by image plate recording at the CHESS F1 synchrotron beam line. These data will be used to generate a refined structure in the near future.

Projects for which the new data collection procedures will be especially important are structural analyses of rotavirus single-shelled particles (SSPs) and reovirus cores. These are the (probably homologous) internal structures derived from double-strand RNA (dsRNA) viruses. They are transcriptionally active assemblies that synthesize, modify, and

extrude messages from each of the dsRNA segments. They are both ~700 Å in diameter, and their crystals have unit cell dimensions >1,000 Å. Data to ~15-Å resolution have been collected from the crystals of rotavirus SSPs, and observed diffraction extends to at least 5 Å. Efforts are under way to compute the low-resolution structures of these particles.

The envelope protein of tick-borne encephalitis virus (TBE) is the principal viral antigen, and it is believed to mediate receptor binding and membrane fusion. A dimeric soluble fragment (polypeptide M_r ~ 45,000), comprising ~80% of the molecule, can be released from virions by proteolytic cleavage. Crystals of this soluble form diffract to 2.6-Å resolution. A low-resolution (5-Å) electron density map shows the overall shape of the subunit. Work is in progress to extend the resolution of the phase determination, in order to build a complete model of the folded polypeptide chain.

Receptors

The endocytosis of receptors for proteins such as growth factors, transferrin, and LDL (low-density lipoprotein) involves directed uptake by coated vesicles, transport to internal compartments where ligand dissociation and sorting can occur, recycling to the cell surface in some cases, and delivery to lysosomes in others. The transferrin receptor (tfR), one of the best characterized of the proteins that participates in this pathway, is a homodimer of 90-kDa subunits.

Crystals of a 70-kDa tryptic fragment, termed tfR-t, which represents nearly all of the extracellular part of tfR, will allow molecular details of such a receptor to be visualized. Substantial progress has been made during the past year toward determining the tfR-t structure. Crystals grown from tfR-t derived from placental preparations diffract to at least 3.5-Å resolution, but radiation sensitivity and a large unit cell make data collection difficult. Accurate diffraction data can now be measured, using "frozen" crystals at −170°C and high-intensity synchrotron beams, and several excellent data sets have been recorded.

Expression of a recombinant, soluble receptor, corresponding almost precisely to the tryptic fragment, has also just been accomplished. The product, secreted in good yield from CHO cells, is dimeric and binds transferrin. Its availability should substantially facilitate the crystallographic studies.

Crystals of the complete "four-domain" extracellular segment of the T cell co-receptor CD4 are also difficult to study—probably because CD4, like tfR, is an elongated molecule that packs awkwardly into a very large crystalline unit cell. Again in this case,

low-temperature data collection has proved to be crucial for accurate diffraction measurements. The structure of a two-domain fragment was reported by Dr. Harrison's group in 1990, and a picture of the complete extracellular segment will be important for understanding the binding of CD4 to class II MHC (major histocompatibility complex) molecules. (The CD4 project is supported by grants from the National Institutes of Health.)

DNA-binding Proteins for Transcriptional Regulation

The yeast transcriptional activator GCN4 has a segment at its carboxyl terminus that forms a so-called basic region/leucine zipper (bZip) element, which governs dimerization and DNA binding. It is unfolded at low concentration, but the presence in free solution of specific DNA induces an almost completely α-helical structure. Work in other laboratories has shown that the carboxyl-terminal part of the element (the leucine zipper) forms a dimeric α-helical coiled coil when this transition occurs.

A crystal structure of the GCN4 bZip fragment, specifically bound to DNA, shows that essentially the entire 58-residue fragment forms a continuous α helix. The part corresponding to the basic region diverges gently from the carboxyl-terminal coiled coil, and each basic region in the dimer traverses a DNA major groove on either side of the center of the binding site. The entire complex has the appearance of a molecular tweezers grasping the DNA.

A set of five residues in the basic region, largely conserved across the family of bZip proteins, specifies the sequence of the binding site. Indeed, many of the related proteins (such as the fos/jun heterodimer) bind to similar DNA sequences, and work on crystals of a fragment from the fos/jun complex is in progress.

The amino-terminal 225 residues of GCN4, which include an acidic activator region, do not appear to affect the conformation of the bZip portion. The structural properties of the amino-terminal part remain to be explored. (The GCN4 project has been supported in part by funds from the Lucille P. Markey Charitable Trust.)

Another yeast transcriptional activator, GAL4, is a very large protein with a small amino-terminal region that mediates its binding to upstream activating sites (UAS_G). A fragment containing residues 1–65 has been crystallized in complex with DNA containing a UAS_G sequence, and the structure has been analyzed at 2.7-Å resolution. The protein binds as a dimer to the symmetrical 17–base pair sequence of the UAS_G site. A Zn^{2+}-containing domain (residues 8–40), termed the recognition module, specifies a

conserved CCG triplet at each end of the site through contacts to bases in the major groove. Nuclear magnetic resonance (NMR) studies show that this module is the only part of the 1–65 fragment that is folded when free in solution. The polypeptide-chain backbone of the module has a remarkable internal dyad, but the amino acid sequence only reflects the repeat in the position of Zn-liganding cysteine residues.

Dimer contacts in the complex are provided by a short, coiled coil dimerization module (residues 50–65). This dimer surface is known to be augmented when additional residues are present. Recently grown crystals of a longer fragment from the related PPR1 regulatory protein should reveal a more complete dimerization element. A segment of extended polypeptide chain links the recognition module to the dimerization element and specifies the length of the site. The relatively open structure of the complex would allow another protein to bind coordinately with GAL4. (The GAL4 project is supported by a grant from the National Institutes of Health.)

Dr. Harrison is also Professor of Biochemistry and Molecular Biology and Research Associate in the Laboratory of Molecular Medicine at the Children's Hospital, Boston.

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STRUCTURAL STUDIES ON BIOLOGICAL MACROMOLECULES

Wayne A. Hendrickson, Ph.D., *Investigator*

Dr. Hendrickson and his co-workers study the structure and biological action of macromolecules, using diffraction analysis and other biochemical and biophysical methods as their principal research tools. They combine specific structural studies on important biological problems with methodology development aimed at facilitating their investigations. The methodology work features synchrotron radiation, diffraction methods, and computational crystallography. The main biological themes concern cell surface interactions, genetic replication, carbohydrate-mediated recognition, and oxygen transport. The studies are also directed at general principles of protein structure, dynamics, and assembly.

Cellular Signal Transduction

The responses of cells to their environment obviously involve molecular interactions at their cell surfaces, and many of these events generate signals that traverse the cell membrane. Crystallographic studies on extracellular receptor fragments and on intracellular kinase fragments and other transducing factors are beginning to elucidate some of the molecular principles governing these processes. During the past year, efforts have continued in the Hendrickson laboratory on immune system receptors, on growth factors and their receptors, and on protein kinases; studies have also been initiated on cell adhesion molecules.

Regarding CD4, a second crystal form of the D1D2 fragment has been solved; crystals have been grown from the first of a series of mutant proteins (with Dr. Ray Sweet, SmithKline Beecham); and the CD4:class II interaction is being studied in structure-guided mutational studies (with Dr. Rafick Sekaly, Clinical Research Institute of Montreal). The crystal structure of an extracellular fragment of CD8 has

been refined and analyzed (with Dr. Richard Axel, HHMI, Columbia University). Structure determinations are now in progress for various growth factors, including stem cell factor (with Amgen) and ciliary neurotrophic factor (with Regeneron). Crystallization efforts continue on extracellular fragments of the insulin receptor (with Dr. Joseph Schlessinger, New York University) and on other receptors.

The group also continues its attempts to express and crystallize kinases, including proteolytically defined fragments of the insulin receptor (with Dr. Leland Ellis, Texas A&M University, Houston), protein kinase C (with Dr. Paul Kirschmeier, Schering-Plough), and the lymphocyte kinase p56^{lck}. In addition, x-ray absorption spectroscopy has been used (with Dr. Steven Cramer, University of California, Davis) to characterize zinc sites in protein kinase C. Finally, an initiative on cell adhesion molecules has yielded the crystal structure of an adhesive fibronectin type III domain from tenascin (with Dr. Harold Erickson, Duke University) and crystals of a four-domain fibronectin fragment (also with Dr. Erickson), of the myelin sheath adhesin p0 (with Dr. David Coleman, Columbia University), and of a cadherin fragment (also with Dr. Coleman).

Replication and Transcription

Macromolecular interactions involved in the regulation and catalysis of genetic replication and transcription are at the heart of molecular biology. The Hendrickson group is also involved in studies in this area. Following on the previously reported structure of ribonuclease H (RNase H) from *Escherichia coli,* inactive site-directed mutants have been produced, crystal structures solved, and complexation with RNA:DNA hybrids demonstrated. The RNase H protein of reverse transcriptase (RT) from Moloney murine leukemia virus has been expressed (with Dr. Stephen Goff, Columbia University); a proteolytic fragment of the polymerase portion of Moloney RT has also been produced (also with Dr. Goff); and crystals of the selenomethionyl analogue of the polymerase fragment have been prepared for the structure determination. Work has also proceeded on the crystallographic analysis of the *umuD'* mutation-repair protein from *E. coli* (with Dr. Roger Woodgate, National Institutes of Health) and on *Taq* polymerase (with Dr. David Sharkey, Eastman-Kodak).

Carbohydrate Recognition

The highly glycosylated surfaces of cells help to confer a distinctive character, and the identity of these carbohydrate labels can be recognized by specific animal lectins. A large class of such lectins are characterized by calcium-dependent (C-type) car-

bohydrate recognition domains (CRDs). Crystals of a dimeric CRD fragment from mannose-binding protein, an antibody-independent defense agent, have been analyzed both as the Ho^{3+} complex and as a Ca^{2+} complex with a six-mannose glycopeptide (with Dr. Kurt Drickamer, Columbia University). The structures show that specific binding is dictated by direct coordination to a calcium site that involves displacement of a water ligand by two sugar hydroxyl groups. Structural work is also proceeding on a Fab fragment of antidextran antibody (with Dr. Elvin Kabat, Columbia University).

Other Structural Programs

Work continues on other problems of long-standing interest to this group, including oxygen-carrying proteins, metalloproteins, and streptavidin. The copper sites have been located for a fragment of octopus hemocyanin (with Dr. Kensal van Holde, Oregon State University); structures have been refined for streptavidin complexed with various biotin analogues; *Pyrularia* thionin, a relative of crambin, has been crystallized (with Dr. Leo Vernon, Brigham Young University); and the structure determinations for two isoforms of β-bungarotoxin are nearing completion.

Methodology Development

Work on methods is an integral aspect of many of the structural projects in Dr. Hendrickson's laboratory. The problem of crystallographic phase determination remains an important focus of attention, and with support from the National Institutes of Health, applications of anomalous scattering are being explored in this context.

During the past year a number of extensions have been made in methods for multiwavelength anomalous diffraction (MAD). These include a statistical test for enantiomorph selection, improved procedures for the efficient bacterial incorporation of selenomethionine into proteins, and the development of MAD phasing procedures, with data restricted to wavelengths very near absorption edges. The problem of refining atomic models into optimal agreement with diffraction measurements is another major area of interest, and with support from the National Science Foundation, procedures are being developed for the economical incorporation of dynamic information into macromolecular refinement.

A third area of methodology development concerns crystallographic computing. A convenient windowing interface to the many computer programs needed in these studies is being developed as the Crystallographic Workbench. With support

from the National Science Foundation to Dr. Bourne, an object-oriented query system is also being developed to interface with the Protein Data Bank. Finally, the second annual book of illustrated abstracts of all new x-ray and NMR (nuclear magnetic resonance) structures has been produced (with Kurt Wüthrich, ETH Zurich).

HHMI Synchrotron Resource

The HHMI facility for synchrotron radiation at the National Synchrotron Light Source at Brookhaven National Laboratory is nearly ready for its first phase of routine use. With the assistance of a Technical Advisory Committee appointed during the past year, the commissioning of the first beam line for MAD experiments is nearing completion, and a program has been established to complete the second beam line for routinely rapid data measurements during the next year. Procedures are being developed to assure rapid access and convenient use of these facilities by HHMI and other investigators.

Dr. Hendrickson is also Professor of Biochemistry and Molecular Biophysics at Columbia University College of Physicians and Surgeons.

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X-RAY DIFFRACTION STUDIES OF PROTEIN STRUCTURE AND FUNCTION

John Kuriyan, Ph.D., *Assistant Investigator*

Current research in Dr. Kuriyan's laboratory is aimed at obtaining atomic-level descriptions of how proteins carry out their specific functions. The emphasis is on determination of three-dimensional structure by x-ray crystallography and on subsequent computer simulation of protein structure and dynamics. Projects are under way in three broad areas: DNA-replication proteins, *src*-related oncogene products, and redox enzymes (the last with support from the National Institutes of Health). In addition, methodological work aimed at improving the x-ray structure refinement process is also being carried out, with support from the National Institutes of Health.

In collaboration with Dr. Michael O'Donnell (HHMI, Cornell University) and Rene Onrust, the β subunit of the *Escherichia coli* DNA polymerase III complex (PolIII) has been crystallized and its three-dimensional structure determined to 2.5-Å resolution by x-ray crystallography. DNA polymerases are enzymes that duplicate the information content of DNA by catalyzing the template-directed polymerization of nucleic acids. Polymerases that are involved in chromosomal replication, such as PolIII, are distinguished by their high processivity; i.e., they can perform rapid replication (1,000 bases/s) of very long stretches of DNA without dissociation.

This property is conferred upon PolIII by the β subunit, which acts to clamp the polymerase onto DNA. The β subunit is bound very tightly to DNA. Once assembled on circular duplex DNA, for example, it cannot be readily dissociated. It is, however, nonspecific in its interactions with DNA and has been shown to move along duplex DNA freely.

How does the β subunit manage to bind DNA with both tight (nondissociative) and loose (nonspecific) interactions? The x-ray structure reveals that two molecules of the β subunit are tightly associated to form a donut-shaped structure that can encircle DNA. This unprecedented molecular topology is surprisingly symmetric. Each monomer consists of three domains of identical chain topology. Each of these is roughly twofold symmetric, with an outer layer of two β sheets providing a scaffold that supports two α helices. Replication of this motif around a circle results in a rigid molecule with 12 α helices lining the inner surface of the ring and with six β sheets forming the outer surface.

The high symmetry of the ring-like structure is well suited to slide along cylindrically symmetric DNA. Structural analysis of the *E. coli* protein suggests that eukaryotic DNA polymerases have processitivity factors with similar architecture. These proteins are known as proliferating cell nuclear antigens (PCNAs). Crystals of yeast PCNA that diffract to 3-Å resolution have been obtained in collaboration with Dr. Peter Burghers (Washington University). Structural analysis of PCNA and higher-resolution studies of the β subunit are in progress.

The laboratory is collaborating with various groups to purify and crystallize *src*-related oncogene products and their cellular equivalents. Initial efforts were focused on the SH2 domain of the v-*src* tyrosine kinase, in a collaborative effort with the laboratories of Drs. Hidesaburo Hanafusa, David Cowburn, and David Baltimore (Rockefeller University) and Dr. Marilyn Resh (Sloan-Kettering Institute). SH2 domains are modular units that bind to phosphorylated tyrosine residues and are found in many proteins involved in signal transduction. Most proteins that contain these domains also contain other modules with various catalytic or binding activities, and the SH2 domains serve to localize these diverse signal transduction proteins at different sites of tyrosine phosphorylation, such as activated growth hormone receptors.

Mutations in SH2 domains have been shown to affect the cellular transforming properties of various oncogenes, and there is great interest in designing specific inhibitors that may modulate these properties. About 30 different SH2 domains have been identified, and sequence analysis suggests that they are likely to share a common molecular architecture. However, they do not show sequence similarity with any proteins of known three-dimensional structure. This has prevented the application of computer modeling methods to model and predict their structure.

The v-*src* SH2 domain has been overexpressed, purified, and crystallized in the laboratory. High-resolution structures of the SH2 domain complexed with two phosphotyrosyl peptides have been determined by x-ray crystallography. These structures have shown that the SH2 domain has a novel architecture for binding peptides and have provided the first view of phosphotyrosine-protein interactions. The recognition of the phosphotyrosine group by the SH2 domain involves unusual interactions wherein positively charged groups of the protein interact with both the negatively charged phosphate

group as well as the ring system of the tyrosine. These structures have set the stage for modeling the structure of other SH2 domains and for designing mutations and inhibitors that affect SH2 function.

The laboratory is also continuing work on redox enzymes, with funding from the National Institutes of Health. Thioredoxin and proteins closely related to it (protein disulfide isomerases) have been implicated in the process by which disulfide-containing proteins fold up rapidly without scrambling their disulfide pairings. Drs. James Bardwell, Karen McGovern, and Jon Beckwith (Harvard Medical School) have recently identified an *E. coli* protein that is required for the correct folding of disulfide-containing proteins *in vivo*. This 21-kDa protein, the product of the *dsbA* gene, has no significant sequence similarity to any protein of known three-dimensional structure, except for a short stretch of amino acids related to the active site of thioredoxin (including the redox-active disulfide bond).

Knowledge of the three-dimensional structure of the protein will aid in understanding its mechanism of action and will reveal whether dsbA belongs to the thioredoxin family. The protein has been purified in the laboratory, and single crystals that diffract to 2-Å resolution have been obtained. A variant dsbA that contains selenomethione instead of methione has been produced and crystallized. This will allow the application of the multiwavelength x-ray phasing technique for the determination of the structure.

Dr. Kuriyan is also Associate Professor and Co-Head of the Laboratory of Molecular Biophysics at the Rockefeller University.

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STRUCTURAL BASIS OF INTERACTIONS WITHIN AND BETWEEN MACROMOLECULES

Brian W. Matthews, Ph.D., D.Sc., *Investigator*

Dr. Matthews and his colleagues use x-ray crystallography, in concert with other techniques, to address some fundamental problems in biology: How do proteins spontaneously fold into their biologically active three-dimensional configurations? What determines the stability of these folded proteins? Can stability be improved? How do proteins interact with each other? How do proteins interact with DNA? How do enzymes act as catalysts?

The Protein-folding Problem

An area of long-standing interest is the so-called protein-folding problem. How does a newly synthesized, extended peptide chain "know" how to fold spontaneously into its active three-dimensional shape?

Although it has long been recognized that the amino acid sequence of a protein determines its three-dimensional structure, recent work from sev-

eral laboratories has revealed that certain amino acids are more important than others in the folding process. At some positions, typically the solvent-exposed mobile sites in the folded protein, amino acids can be interchanged almost at random with little apparent effect on folding or stability. On the other hand, interchange of amino acids in buried or rigid parts of a folded protein can destabilize it, suggesting that the amino acids at these positions are important in determining the folded conformation.

To try to simplify the complexity of the protein-folding problem, Xue-jun Zhang and Dr. Dirk Heinz have attempted to replace "nonessential" amino acids in bacteriophage T4 lysozyme with alanine. Such a "polyalanine protein" would, in principle, truncate all nonessential side chains and bring into focus those parts of the amino acid sequence that are critical to the folding process.

In experiments to date, a series of alanines has been introduced within two different α helices of T4 lysozyme. The somewhat surprising result is that alanines are not only tolerated at most positions in the α helix, they can sometimes increase the protein's stability. In an extreme case it has been found that 10 alanines can be introduced in sequence, yet the protein still folds normally and has full activity. This illustrates that the information in the amino acid sequence of a protein is highly redundant.

Understanding the Interactions That Stabilize Protein Structures

It is generally agreed that the major factor in stabilizing the folded structures of globular proteins is the hydrophobic effect. A principal difficulty in quantitating this effect has been the lack of relevant structural data. How does a protein structure respond when a bulky hydrophobic residue such as leucine is replaced by a smaller residue such as alanine? Does the protein structure remain essentially unchanged or is it rearranged to avoid the creation of a cavity? If cavities are created, do they contain solvent?

To address these questions Dr. Elisabeth Eriksson constructed cavity-creating mutants in which a large hydrophobic amino acid was replaced by a smaller one within the hydrophobic core of bacteriophage T4 lysozyme. Several such variants were crystallized and the structures determined at high resolution. The structural consequences of the mutations differed from site to site. In some cases the protein structure hardly changed at all. In other cases, both side-chain and backbone shifts up to 0.8–1.0 Å were observed. In every case removal of the wild-type side chain allowed some of the surrounding atoms to move toward the vacated space, but a cavity always remained.

This suggests a way to reconcile the different values for the apparent strength of the hydrophobic effect. Two extreme situations can be imagined. In the first, a leucine → alanine replacement is constructed, and the protein structure remains completely unchanged. Here the size of the created cavity is large, and the mutant protein is maximally destabilized. In the other extreme, the protein structure relaxes in response to the leucine → alanine substitution, fills the space occupied by the leucine side chain, and so avoids the formation of any cavity whatsoever. In this case the decrease in energy of the mutant protein relative to the wild type drops to a constant energy term that is characteristic for a leucine → alanine replacement.

Ligand Binding Within Cavities

Dr. Eriksson and Dr. Walt Baase also showed by crystallographic and thermodynamic analysis that the cavity created by the replacement leucine 99 → alanine in T4 lysozyme is large enough to bind benzene and that ligand binding increases the melting temperature of the protein by 5.7°C. This shows that cavities can be engineered within proteins and suggests that such cavities might be tailored to bind specific ligands. The binding of benzene at an internal site 7 Å from the molecular surface also illustrates the dynamic nature of proteins, even in crystals.

Receptor-Ligand Interaction

To develop an understanding of the mode of action of growth factors and their interactions with their receptors, Dr. Eriksson and Dr. Larry Weaver, in collaboration with Dr. Lawrence Cousens (Chiron Corp.), crystallized, determined, and refined the high-resolution structure of human fibroblast growth factor. The structure is very similar to that of interleukin-1β. Clearly, many growth factors have similar overall structures, but the exact relationship of these factors in the vicinity of their receptor-binding regions remains to be clarified.

Protein-DNA Interaction

Dr. Matthews and his colleagues have been interested for some time in the interaction between proteins and nucleic acids. In 1981 they determined the structure of the Cro repressor protein of λ bacteriophage (bacteria-infecting virus). Cro has served as one of the prototypical examples of a DNA-interacting protein. Drs. Richard Brennan and Steven Roderick, in collaboration with Dr. Yoshinori Takeda, have also determined the crystal structure of Cro in complex with a tight-binding 17–base pair DNA operator and are improving the accuracy of

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the structure by a process of crystallographic refinement.

Drs. Keith Wilson and Lisa Shewchuk, and Dr. Richard Brennan in collaboration with Dr. Anthony Otsuka, have recently determined the three-dimensional structure of the biotin repressor from *Escherichia coli*. This DNA-binding protein is both an enzyme and a repressor of transcription. The structure includes a helix-turn-helix DNA-binding region and a separate domain containing the enzymatic active site.

Studies of protein stability and protein-DNA interaction were supported in part by grants from the National Institutes of Health.

Dr. Matthews is also Professor of Physics at the University of Oregon, Eugene, and Adjunct Professor of Biochemistry and Molecular Biology at the Oregon Health Sciences University.

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STRUCTURE AND DESIGN OF DNA-BINDING PROTEINS

Carl O. Pabo, Ph.D., *Investigator*

Dr. Pabo's research has focused on the structure and design of DNA-binding proteins. The laboratory is attempting to understand how proteins recognize specific sites on double-strand DNA and how the bound proteins regulate gene expression. During the past two years, Dr. Pabo's laboratory has revealed how some of the major families of regulatory proteins recognize their binding sites. This information will eventually be used to help design novel DNA-binding proteins for research, diagnosis, and therapy.

Crystallographic Studies
of Repressor-Operator Interactions

Prokaryotic repressors provide useful model systems for the study of protein-DNA interactions, and Dr. Pabo's laboratory has been studying the repressor from bacteriophage λ. Several years ago the laboratory solved the crystal structure of a complex that contains the DNA-binding domain of the repressor and a 20–base pair operator site. This structure showed how the prokaryotic helix-turn-helix (HTH) motif is used for DNA recognition. Crystallographic refinement at high resolution has now revealed further details about the repressor-operator interactions and has shown how an extended amino-terminal arm makes additional contacts in the major groove. (The project described above was supported by a grant from the National Institutes of Health.)

Crystal Structures
of Homeodomain-DNA Complexes

The homeodomain is a conserved DNA-binding motif that is found in a large number of eukaryotic regulatory proteins. Although the intact proteins usually are much larger, the homeodomain itself contains about 60 amino acids. Several hundred homeodomains, which bind to closely related DNA sites, have been sequenced. They provide an interesting and important system for studying protein-DNA interactions.

Over the past several years, Dr. Pabo's laboratory has determined the structures of two homeodomain-DNA complexes. These crystal structures revealed that the homeodomain contains an extended amino-terminal arm and three α helices. Helices 2 and 3 form an HTH unit that is closely related to the HTH unit found in a family of prokaryotic repressors. Although the overall fold of the HTH unit is conserved, helices 2 and 3 of the homeodomain are longer than the corresponding helices in the λ repressor, and they dock against the DNA in a significantly different way. The homeodomain also has an extended amino-terminal arm that makes site-specific contacts in the minor groove.

Even though the two homeodomains (one from yeast and one from *Drosophila*) have different amino acid sequences, the structures of the proteins and the complexes are quite similar. Dr. Pabo's research suggests that all homeodomains bind in fundamentally similar ways, and a general model is proposed for homeodomain-DNA interactions. (The projects described above were supported by a grant from the National Institutes of Health.)

Crystal Structures
of Zinc Finger–DNA Complexes

Another structural motif, the zinc finger domain, has also been found in a large number of eukaryotic DNA-binding proteins. These domains have about 30 amino acids and contain conserved cysteine and histidine residues that bind to zinc. Dr. Nikola Pavletich, a research associate in the laboratory, has determined the structure of two zinc finger–DNA complexes.

Last year he determined the crystal structure of a zif268 complex that contains three zinc fingers and a consensus binding site. (The immediate-early mouse gene *zif268* was characterized by Dr. Daniel Nathans [HHMI] and his colleagues at the Johns Hopkins University.) This structure revealed that each finger docks against the DNA in a similar way and contacts a 3–base pair subsite. In each finger, residues near the amino-terminal end of an α helix make critical contacts in the major groove. The repeating, modular arrangement seen in this complex suggested that it might be possible to mix and match different fingers to design DNA-binding proteins with new specificities.

This year Dr. Pavletich determined the structure of a complex that contains the five zinc fingers from the human GLI protein. (This protein, which is amplified in certain glioblastomas, was characterized by Dr. Bert Vogelstein and his colleagues at the Johns Hopkins University.) Although the overall arrangement of the fingers in GLI is similar to that discovered in zif, subtle variations in the position and orientation of the fingers allow a variety of new side chain–base interactions. Comparing the zif and GLI complexes helps explain how zinc fingers can be used to recognize so many different binding sites.

Once again, these studies suggest that zinc fingers will provide a powerful and adaptable framework for the design of novel DNA-binding proteins.

Strategies for Designing DNA-binding Proteins

The laboratory is also developing strategies for computer-aided protein design and for genetic selection of DNA-binding proteins with novel binding specificities. The strategies for protein design are implemented in a package of programs and subroutines known as PDB-PROTEUS. These programs, which include simple methods for characterizing spatial relationships, are being used to compare side chain–base interactions and side chain–phosphate interactions in all known protein-DNA complexes. This systematic analysis of spatial relationships should help explain the structure and evolution of DNA-binding proteins and should provide a firm foundation for designing new regulatory proteins.

Dr. Pabo's laboratory is also testing several methods for experimentally screening large numbers of sequence variants. One strategy involves expressing DNA-binding proteins on the surface of the fd bacteriophage. This may allow the use of affinity chromatography to isolate DNA-binding proteins with novel specificities, and these screening and selection strategies should complement computer-aided protein design.

Dr. Pabo is also Professor of Biophysics and Structural Biology in the Department of Biology at the Massachusetts Institute of Technology.

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PROTEIN STRUCTURE, FUNCTION, AND MOLECULAR RECOGNITION

Florante A. Quiocho, Ph.D., *Investigator*

The interaction between a protein and its ligand, large or small, forms the basis of biological specificity. It is responsible for the remarkable selectivities exhibited, for example, by enzymes for their substrates, by transport proteins for their nutrients, by binding proteins for their ligands (e.g., DNA, metals), and by antibodies for their antigens. Dr. Quiocho and his colleagues are studying the three-dimensional structures of a number of proteins in order to understand a variety of protein-ligand interactions at the atomic level. Although x-ray crystallography is the principal experimental approach, other correlative techniques—biochemical, physicochemical, and recombinant DNA—are also employed.

Calmodulin: Target Enzyme Recognition

Calmodulin (CaM) is the key calcium-dependent regulator of a variety of eukaryotic intracellular processes. In many of these the regulator activates more than 20 enzymes, and the CaM-binding domains in several of these enzymes have been shown to reside in a region consisting of an 18-residue peptide segment. Although these domains show considerable sequence diversity, most are predicted to form amphipathic helices. Synthetic peptides based on the CaM-binding domains of the target proteins have been shown to bind CaM in a calcium-dependent manner with the same stoichiometry and affinity as the native protein.

To understand the atomic details of the mechanism by which CaM recognizes and binds to target proteins, the laboratory has recently determined two structures. First, the structure of native CaM has been refined to 1.7-Å resolution. This is a higher resolution structure than those of several other investigators. The native structure, like all others determined previously, reveals a dumbbell-shaped molecule in which two structurally similar globular domains, each containing a pair of calcium-binding

sites, are widely separated by an eight-turn, solvent-exposed, central α helix.

Second, the laboratory has also determined and refined (to 2.4 Å) the structure of CaM bound to a 20-residue peptide analogue of the CaM-binding region of a target enzyme, smooth muscle myosin light-chain kinase. In the largest conformational change ever observed for a protein, the binding of CaM to the target peptide causes a five-residue turn of the central α helix to unwind and expand into a bend, such that the two domains converge and wrap around the α-helical peptide. Going from the structure of the unbound CaM to that of the CaM-peptide complex amounts to an $\sim 100°$ bend and $\sim 120°$ twist between the two domains.

The close association of the two domains creates a continuous hydrophobic patch that encompasses the shallow hydrophobic pocket in each domain. This patch interfaces with the hydrophobic side of the α-helical peptide. Of the ~ 185 contacts (< 4 Å) formed between CaM and the peptide, 80% are van der Waals contacts, 15% hydrogen bonds, and 5% salt links. The overwhelming involvement of van der Waals forces helps explain why CaM forms complexes with so many target proteins that show little sequence similarity in their CaM-binding domains.

Adenosine Deaminase: Mimics of Pretransition and Transition States

Adenosine deaminase (ADA; $M_r = 40,000$), a key enzyme in purine metabolism, catalyzes the irreversible hydrolysis of adenosine or deoxyadenosine to their respective inosine product and ammonia. It has a central role in maintaining immune competence; lack of the enzyme is associated with severe combined immunodeficiency disease (SCID).

The determination of the x-ray structure of adenosine deaminase, reported last year, led to the discoveries that it contains a zinc cofactor and that the bound ligand is 6R-hydroxyl-1,6-dihydropurine ribonucleoside (HDPR), a nearly ideal transition-state analogue, rather than purine ribonucleoside, which cocrystallized with the enzyme.

The structure of ADA in complex with 1-deazaadenosine, an almost perfect substrate or ground-state analogue, has recently been determined and refined at 2.4-Å resolution. This complex is considered to be a mimic of the pretransition state. The structure of the complex revealed the presence of a zinc-activated water or hydroxide. These structures, and the complex with the HDPR transition-state analogue, have provided a detailed anatomy of the chemical reaction catalyzed by ADA. The requirement of a zinc-bound water molecule is firmly established. Also, there is little doubt that a zinc-activated water (incipient hydroxide) is the nucleophile attacking the C6 of adenosine.

The structure determination of the complex of ADA with inosine, the product of the enzyme-catalyzed reaction, is almost finished.

Antibody Recognition of the O-Antigen of *Shigella flexneri* Polysaccharide

The laboratory has determined and refined at 2.5 Å the structures of the Fab fragment of the antibody, alone and in complexes with two oligosaccharide antigens: the trisaccharide α-Rha(1-3)α-Rha(1-3)β-GlcNAc and the pentasccharide α-Rha(1-2)α-Rha(1-3)α-Rha(1-3)β-GlcNAc(1-2)α-Rha. The antigenic site is designed to recognize only a trisaccharide. This structural work is in line with the interest of the laboratory in protein-carbohydrate interactions. Moreover, this work is relevant, as oligosaccharide epitopes of bacterial and tumor cell surface are considered to be disease markers and targets for therapeutic antibodies.

Signal Transduction in Periplasmic Receptors for Active Transport and Chemotaxis

Ligand-induced conformational change of the periplasmic receptors of bacterial cells plays two important roles. It enables ligand recognition and affinity to be fully achieved and at the same time confers the precise geometry for productive docking of the periplasmic receptors with the membrane-bound components. Determining by x-ray crystallography the structures of both the ligand-free and ligand-bound structures of the receptor for maltodextrins has provided a precise understanding of the nature of this conformational change. Binding of maltodextrins to the receptor causes a large hinge-bending and a small twist motion between the two domains, bringing them together and enclosing the ligand bound in the cleft between them. It is this closed liganded form that is presumably preferentially recognized by the membrane-bound components, thus triggering the signaling mechanism that initiates transport or chemotaxis.

Dr. Quiocho is also Professor of Biochemistry and Structural Biology and of Molecular Physiology and Biophysics at Baylor College of Medicine.

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MULTIDIMENSIONAL CHROMOSOME STRUCTURE

John W. Sedat, Ph.D., *Investigator*

The research of Dr. Sedat and his group is aimed at a structural understanding, with functional correlations, of the interphase chromosomes in the eukaryotic nucleus. Three-dimensional structure is analyzed at the cellular and subcellular levels. The approach involves data collection and computer processing at the resolution limits of optical and electron microscopy.

In a complementary collaboration with Dr. David Agard (HHMI, University of California, San Francisco) and his colleagues, the group seeks answers to these questions: How do interphase chromosomes fold in the intact diploid nucleus; and how, in detail, does an interphase chromosome change as a function of progression through the cell cycle and development? What is the defined interphase chromosome architecture of a specific gene? Is molecular information reflected in characteristic structural attributes? The groups continue to use *Drosophila melanogaster* for these integrated structural and functional studies.

Nuclear and Chromosomal Structural Dynamics: Multidimensional Studies

Studies of the architecture of chromosomes include efforts to understand the role of specific proteins in chromosomal organization and dynamics. A powerful approach is to study structural dynamics in the living cell. As a first step, fluorescently labeled proteins or labeled monoclonal antibody Fab fragments (MCA-F) are injected into the *Drosophila* embryo, producing, under optimal conditions, little or no perturbation of development.

These laboratories have described in recent years the development of multidimensional optical microscopy. They now observe routinely the three-dimensional distribution of distinct cellular components every 20–30 seconds (five-dimensional microscopy: three-dimensional space + time + cellular component). The time resolution should increase ~10-fold when digital camera and hardware development are completed in the next few months. The laboratories continue to develop a computer-based approach to the quantitative extraction and analysis of large- and small-scale motions of chromosomes and other structures within the nucleus.

This methodology has been used to study the *in vivo* distribution and dynamics of topoisomerase II (topoII), an enzyme required for chromosome condensation and segregation. Three-dimensional time-lapse studies using embryo-injected fluorescently labeled topoII (or labeled topoII-specific MCA-F) have shown that the concentration of nuclear topoII changes dramatically throughout the cell cycles. There are at least three pools of topoII. During pro-

phase, ~60% of the enzyme present in the late interphase nucleus leaves it and diffuses into the cytoplasm. The remaining 40% is associated with the chromosomes throughout prometaphase and metaphase. After anaphase, 25% of the metaphase topoII leaves the chromosomes.

Control four-dimensional studies, using fluorescently labeled histones as chromosomal labels, have shown that the topoII loss, at specific times, is not the result of chromosomal condensation or segregation. These movies also show that topoII localizes, with temporal regulation, to specific sites within the interphase nucleus. Metaphase chromosomes do not have an axial core along the chromosome length. The localization of the enzyme is thus highly regulated, spatially and temporally, revealing novel behavior as well as a role in chromosome condensation and segregation.

A comparable four-dimensional study was undertaken to dissect, structurally and functionally, the nuclear envelope (NE). Dr. Sedat and his co-workers have shown that the major NE structural protein, lamin, is organized as a highly discontinuous fibrillar network, leaving in the nuclear periphery large voids containing little or no lamin. They have also correlated this structure with the underlying chromatin.

To study the dynamics of the lamins during mitosis, fluorescently labeled lamin-specific MCA-F, together with labeled histones (to follow chromosomes), were injected into early *Drosophila* embryos. A highly discontinuous lamin network was observed *in vivo*, with interlamin fiber spacings approximating 1 μm. New four-dimensional data show a surprisingly complex series of NE and lamin structural dynamics. Laminar structures do not completely disassemble at the onset of mitosis, but persist until well into mitosis. Further lamin structural changes, including NE breakdown and reassembly, take place at anaphase and telophase. Additional studies indicate that lamins are essential for the nuclear structural reorganizations that occur at all points of the cell cycle.

Homologous Chromosome Pairing: Three-Dimensional Nuclear Studies

It has long been debated whether interphase chromosomes follow ordered paths, whether there are special associations between the homologous chromosomes in diploid nuclei, and what roles such associations might play in regulating nuclear organization and function. This group's development of a three-dimensional *in situ* hybridization technique, which allows localization of specific chromosomal sequences under conditions of chromosome structure preservation in intact tissues, provides a new avenue for exploring these questions.

The issue of homologous association has remained particularly significant in *Drosophila* biology, because genetic evidence has shown that expression of certain alleles of a few genes (such as *bx-c*, *dpp-c*, and *sgs-4*) can be affected by the allelic state of the homologous locus. These genetic effects, which appear to depend on interactions in trans between homologous sequences, have been grouped into the phenomenon known as transvection.

The locations of chromosomal loci on the two homologues were probed by *in situ* hybridization to chromosomal DNA in whole-mount embryos, using a DNA sequence specific for the histone gene cluster. Up to the nuclear cycle 13, homologous loci of the cluster were separated during syncytial mitoses. In dramatic contrast, at cycle 14, homologues at the site were found to be associated at a high frequency. Furthermore, the three-dimensional location of the histone gene cluster at the 13th cycle is in a defined plane about halfway between the apical and basal sides of the nucleus, but changes toward a position in the apical side at the 14th cycle, when transcription and differentiation start. It is intriguing that chromosome reorganization is concomitant with profound changes in embryonic development.

The group's recent investigations of homologue associations have focused on single-copy loci, as examples, the *engrailed* and *Bithorax* genes or *Responder of SD*, a heterochromatic locus. Use was made of new procedures to label the DNA probes fluorescently, making possible direct multiple-label *in situ* experiments. Improved hybridization protocols now allow chromosomal probes as small as 12 kb to be detected, very reproducibly, with high signal-to-noise ratios. Each of these loci is also in a spatially defined nuclear plane but is homologously paired much less frequently than the histone locus, a result implying differential control of homologue associations.

To assay homologous chromosome pairing on a whole-chromosome basis, the laboratory is using polymerase chain reaction (PCR) amplification of microdissected polytene chromosomes to develop whole-chromosome DNA probes.

To test whether gene expression and regulation require the association of homologous chromosomes, this group made use of chromosomal rearrangements. In the homozygous LTX13 translocation strains, the association frequency of homologous histone loci stays low, with a perturbed nuclear location at the time of gastrulation, yet these embryos develop normally to fertile adults. Therefore, full homologous association at the time of gas-

trulation is not essential for this locus. A study of additional chromosomal rearrangements is under way.

Analysis of many adjacent nuclei in embryos has revealed that homologously paired nuclei (early in development) or residual unpaired nuclei (late in development) occur in patches, possibly indicating a cell lineage relationship. To analyze these features further, the group continues to develop methodology (in collaboration with Dr. Zvi Kam of the Weizmann Institute) for automatically locating and quantitating *in situ* such patches in thousands of nuclei on whole surfaces of embryos.

Homologous chromosome pairing during meiosis was also investigated, using genetically and molecularly characterized plant maize. A three- and four-dimensional analysis of leptotene-diakinesis chromosome structure is in progress. This is a collaborative study with Dr. Zack Cande and his group at the University of California, Berkeley.

Chromosomal Substructure Determination

Dr. Sedat's laboratory continues to investigate the substructure of defined regions of polytene chromosomes (through a grant from the National Institutes of Health).

Dr. Sedat is also Professor of Biochemistry and Biophysics at the University of California, San Francisco.

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CHEMICAL MECHANISMS IN CELLULAR REGULATION

PAUL B. SIGLER, M.D., PH.D., *Investigator*

Dr. Sigler's laboratory continues to study the mechanisms of two cell regulatory processes: controlled gene expression and transmembrane signaling. The approach is to crystallize the macromolecules in complexes that reveal the stereochemical basis of their function. The mechanisms inferred from these studies are tested by biochemical, physicochemical, and directed mutational studies. Whereas the short-range goal is to visualize the stereochemical mechanisms, the ultimate goal is to describe the physical chemistry of these cellular processes in energetic and dynamic terms.

Transcriptional Regulation: The Chemistry of Specific Protein-DNA Interactions

Crystallographic studies have been undertaken on a range of prokaryotic and eukaryotic regulatory complexes, with a view to explaining in chemical terms how transcription regulators are targeted to their specific DNA segments.

The trp *repressor-operator complex.* The unexpected stereochemistry of the specific interface seen in the high-resolution crystallographic analysis of the *trp* repressor-operator complex provoked the assertion that an incorrect DNA target was cocrystallized. This contention has been refuted and shown to have arisen from experimental artifacts in gel shift analyses.

Two mechanisms of sequence recognition that were given strong credence by this crystal structure have now been experimentally confirmed. The first was that the water molecules could mediate specific interactions. A non-operator sequence designed to provide a water-mediated interaction similar to that seen in the natural complex does, indeed, support specific high-affinity binding of the repressor.

The second mechanism, indirect readout, posits that the target sequence can be more easily deformed than other sequences to assume the noncanonical structure exhibited in the complex. This suggestion has been confirmed in a collaborative study with Dr. Zippora Shakked and Dov Rabinovitch of the Weizmann Institute in Israel. The crystal structure of an uncomplexed DNA decamer contain-

ing the identity elements of the *trp* operator shows the same characteristic pattern of deviations from canonical B-DNA as seen in the complex.

The steroid nuclear receptors. Lipophilic effectors, such as steroid hormones, thyroid hormone, vitamin D, and retinoic acid, regulate the expression of genes through the action of their receptors. These receptors are transcription factors that are targeted as dimers to their respective hormone-responsive genes through small, conserved DNA-binding domains (DBDs). The identity of the DNA target resides in two features: 1) the sequence of two 6-bp half-sites and 2) their arrangement—that is, the number of base pairs that separate the half-sites and whether the half-sites are arranged as inverted or direct repeats.

The structure of the cocrystalline complex formed by the glucocorticoid receptor DNA-binding domain (GR-DBD) and its target showed that the DBD has two zinc finger–like modules that are folded together, one recognizing the half-site and the other providing a strong dimer interface that places the recognition surface of each subunit in register with the opposing half-site. The firm dimer interface required to arrange the recognition surfaces is supported by DNA interactions. Thus DNA supports (if not induces) conformations that are responsible for protein-protein interactions that enable the receptors to recognize their cognate response elements.

The inferences drawn from the glucocorticoid receptor complex have been confirmed by a crystallographic study of a mutagenically derived analogue of the thyroid receptor's DNA-binding domain (TR′-DBD) in a complex where the target's half-sites are separated by 0 bp rather than 3 bp. Changes in the dimer interface no longer require the recognition surfaces of the protein to be separated by 3 bp, and the subunits are rearranged to interact with abutting half-sites.

The E2-enhancer complex from bovine papillomavirus-1 (BPV-1). The papillomaviruses are a family of small DNA viruses that cause hyperplastic epithelial lesions. The most notable pathogens are the human papillomavirus strains HPV-16 and -18, which have been implicated in cervical carcinomas. The products of the E2 gene are pivotal, as they regulate viral transcription from all viral promoters and are essential for viral replication. All forms of the E2 protein are dimeric, and their function requires preferential binding to a highly conserved palindromic target sequence, termed the E2-BS, which occurs 17 times in the viral genome.

Specific DNA-binding and dimerization is mediated through a carboxyl-terminal, 85–amino acid domain that does not include a sequence motif previously associated with DNA binding. The crystal structure of the E2 DNA-binding/dimerization domain (E2-DBD) of BPV-1, bound to a 16-bp idealized E2-binding site, shows a heretofore undescribed dimeric, eight-stranded antiparallel β barrel made up of four strands from each subunit. A pair of α helices symmetrically disposed on the outer circumference of the barrel contain all of the amino acids directly involved in base-sequence recognition. The DNA is severely but smoothly bent around the barrel to enable successive major grooves to engulf these recognition helices. As a result of the very high resolution of the study (1.7 Å), the stereochemistry of the protein-DNA interface is seen in unprecedented detail. The highly conserved recognition surface of the protein and the identity elements of its target form a highly interdependent network of specific interactions.

The arg *repressor-operator.* Arginine co-represses the transcription of arginine biosynthetic genes. The regulatory system has an unusual architecture. The protein is a robust hexamer that binds a tandem of 18-bp *arg* boxes separated by 3 bp. The *arg* repressor also appears to serve as a binding scaffold for a site-specific recombination system. Crystals of the *arg* repressor, both alone and in complexes with *arg* boxes, were grown, and data have been collected to 2.5 Å.

Transmembrane Signaling

Receptor–G protein interaction. The retinal rod provides a well-characterized example of a seven-helical receptor (rhodopsin)–G protein (transducin)–target enzyme (phosphodiesterase) transmembrane-signaling system. Pure bovine rhodopsin, transducin, and arrestin (and proteolytically trimmed fragments thereof) have been prepared in tens of milligram amounts in collaboration with Dr. Heidi Hamm (University of Illinois, Chicago).

A structure determination is currently under way with crystals of a stably activated transducin complex ($G_{t\alpha} \cdot GTP\gamma S$) that diffract usefully to 2.0 Å. The laboratory's objective is to cocrystallize $G_{t\alpha}$ with photoactivated rhodopsin to see directly the molecular mechanism by which the receptor transduces its photoactivation to $G_{t\alpha} \cdot GTP$. The target of the activated $G_{t\alpha} \cdot GTP$ is an oligomeric cGMP phosphodiesterase (PD). The activated transducin activates PD by binding the inhibitory subunit, PDγ. These studies should indicate the structural basis for a G protein's capacity to transduce the activation signal from the receptor to the target enzyme.

Dr. Sigler is also Professor of Molecular Biophysics and Biochemistry at Yale University.

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STRUCTURAL STUDIES OF REGULATORY AND SIGNAL-TRANSDUCTION PROTEINS

STEPHEN R. SPRANG, PH.D., *Associate Investigator*

Three-Dimensional Structures of the TNF Cytokine Family and Their Receptors

Dr. Sprang's laboratory continues its structural studies of members of the tumor necrosis factor (TNF) family of cytokine hormones, which includes TNF-α, synthesized by macrophages, and lymphotoxin (TNF-β), a product of lymphocytes. The cells of individual tissues respond differently to TNF-α and lymphotoxin, depending on their developmental lineage and tissue-specific functions. Systemic manifestations include the induction of the inflammatory response, shock, and cachexia. Both cytokines are directly toxic to certain tumor cell lines. Lymphotoxin is 32% identical to TNF-α in primary sequence and binds to the same receptors.

The laboratory has determined the structures of both TNF-α and TNF-β (research completed by Dr. Michael Eck). The determination of the crystal structure of TNF-β to a resolution of 1.9 Å, with crystals provided by Drs. Mark Ultsch, Abraham DeVos, and Anthony A. Kossiakoff (Genentech), was reported this year. Lymphotoxin is topologically identical to TNF-α, although the structures differ near sites of length variation. Both molecules assemble as trimers, stabilized primarily by hydrophobic amino acids, but the trimer interface of lymphotoxin is notably rich in aromatic residues. The structure of TNF-β is currently being refined with high-resolution data to allow a comparative analysis of trimer stabilization in TNF-α and TNF-β.

Dr. Sprang's laboratory continues its structural studies of the TNF receptor. Several laboratories have cloned two distinct receptors, both of which are capable of engaging TNF-α and lymphotoxin.

Collaborating with Drs. Barbara Brandhuber and Tadahiko Khono (Synergen), the Sprang laboratory has produced crystals of the extracellular domain of the so-called 55-kDa TNF receptor. These crystals belong to the orthorhombic space group P2$_1$2$_1$2$_1$ (a = 77.6 Å, b = 85.1 Å, c = 68.2 Å). These crystals diffract to ~2.8 Å. A second, possibly monoclinic crystal form has also been obtained. Several possible heavy-atom derivatives of the orthorhombic crystals have been identified by Dr. Lynn Rodseth, and data sets for these have been measured.

The extracellular domain of the 55-kDa TNF receptor is the first member of this family to have been crystallized. Work is now in progress to crystallize the complexes between TNF-α or lymphotoxin and either receptor's extracellular domain. The laboratory has isolated stable complexes between TNF-α and the extracellular domain of the 55-kDa receptor by high-performance liquid chromatography (HPLC).

Structure of Basic Fibroblast Growth Factor

Last year the laboratory reported the 1.8-Å resolution structure of basic fibroblast growth factor (bFGF), a polypeptide of 146 residues with mitogenic activity. The factor stimulates the proliferation of many cell types, including fibroblasts, endothelial cells, and myoblasts. The molecule contains a heparin-binding site that anchors it to glycoaminoglycan components of the extracellular matrix and promotes interaction of the factor with its receptor. The structure reveals that FGF belongs to the same structural family as interleukins-1α and -β.

Crystals have now been obtained of a complex between bFGF and a small oligosaccharide analogue

of heparin. Efforts are also under way to prepare crystals of the extracellular domain of the *flg* gene product, which encodes the cellular receptor of FGF. FGF receptor, expressed in SF9 cells, is provided by Dr. Phillip Barr (Chiron).

Crystallographic Analysis of GTP-binding Signal-Transduction Proteins

Hormonal signals induced by growth factors such as those described above are, in many cases, coupled to intracellular transduction systems that involve members of the G protein family. A major interest of the Sprang laboratory has been to define the receptor, effector, and $\beta\gamma$-binding domains of G protein α subunits and to understand the nature of the conformational changes induced by receptor-mediated GTP binding and subsequent GTP hydrolysis. In collaboration with Dr. Alfred Gilman's laboratory (University of Texas Southwestern Medical Center at Dallas), Dr. David Coleman has obtained crystals (space group $P3_12_1$; $a = 80.1$ Å, $b = 106.3$ Å) of the GTP-γS (a nonhydrolyzable analogue of GTP) complex of Giα that diffract beyond 2.4 Å. Gi couples the activation of plasma membrane potassium channels to muscarinic receptors.

Candidate heavy-atom derivatives have been prepared and data collected for solution of the structure by heavy-atom techniques. The laboratory has also prepared crystals of two muteins of Giα. These muteins suffer from well-defined physicochemical lesions in GTP hydrolytic activity or activation. The structures of these molecules will illuminate the mechanisms of catalysis and signal transduction in this important class of proteins.

Crystals have also been obtained of the α subunit of Gs, the heterotrimeric G protein that couples β-adrenergic receptors to adenylyl cyclase. Intense efforts are under way, in collaboration with Dr. Gilman's laboratory, to develop a productive expression system for Gsα to generate sufficient quantities of Gsα for further crystallographic studies.

Mechanism of the Allosteric Transition in Glycogen Phosphorylase

Last year the structure of the activated state of glycogen phosphorylase as induced by adenosine monophosphate was reported (with Drs. Elizabeth Goldsmith, Stephen Withers, and Robert Fletterick). This and ongoing studies have yielded new information about the nature of the allosteric transition in this complex, cooperative enzyme. Previous descriptions of the phosphorylase active state have been extended by the demonstration of domain rearrangements in the tertiary structure of the phosphorylase subunits upon activation. The laboratory is now engaged in structural studies of the activated enzyme with bound substrates and transition-state analogues in order to elucidate the catalytic mechanism of this enzyme, which, despite 40 years of research by several dedicated laboratories throughout the world, remains poorly understood.

Dr. Sprang is also Associate Professor of Biochemistry at the University of Texas Southwestern Medical Center at Dallas.

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MACROMOLECULES OF THE CENTRAL DOGMA OF MOLECULAR BIOLOGY

Thomas A. Steitz, Ph.D., *Investigator*

The laboratory of Dr. Steitz has been using single-crystal x-ray crystallography combined with molecular genetics to elucidate the structure and function of proteins and nucleic acids that are involved in DNA replication, recombination, transcription (and its regulation), and some aspects of protein synthesis. Major achievements of the past year include determination of the structures of 1) a Klenow fragment · DNA complex showing bound duplex DNA and 2) HIV reverse transcriptase complexed with the nonnucleotide inhibitor Nevirapine.

Transcription and Its Regulation

The catabolite gene activator protein (CAP) from *Escherichia coli* is a dimer of 22,500-molecular-weight subunits that activates transcription from certain *E. coli* operons in the presence of cAMP. The crystal structure of this sequence-specific DNA-binding protein shows that each subunit consists of a cAMP-binding domain and a smaller domain involved in binding the DNA.

The crystal structures of CAP cocrystallized with either a 30- or 32-bp DNA fragment have been solved. The most striking result is that the DNA duplex is kinked 43° at two positions in both solved crystals, but the bending at the ends of the DNA differs as a result of differences in crystal packing. Data at 2.8-Å resolution from cocrystals of CAP complexed with a 46-bp fragment should yield a structure in which crystal-packing effects on the end of DNA are different. The role of this CAP-induced DNA bending in transcription activation is being pursued by biochemical and structural studies of CAP and RNA polymerase subunits complexed with promoter DNA.

Recombination

The recA protein of *E. coli* plays a major and essential role in general recombination by catalyzing the formation of three- and four-strand DNA structures that involve homologous pairing. The crystal structure of recA protein has been refined to 2.3-Å resolution. The protein forms the same helical filament in the crystal as seen at far lower resolution in electron micrographs of recA bound to DNA.

Similarities of the ADP binding site to the GTP binding site of the *ras* p21 oncogene have led to hypotheses concerning the allosteric mechanism of coupling between ATP and DNA binding that may be general for many NTPases. Crystals of a recA · ATP analogue complex have been grown, and attempts to crystallize complexes with DNA are being pursued.

The next enzyme in the recombination pathway that cleaves the Holliday junction, ruvC, has been crystallized in a form suitable for structure determination. (These studies on recombination have been supported by the National Institutes of Health.)

DNA Synthesis

Important progress has been made during the past year in refining the structure of Klenow fragment complexed with 11 base pairs of duplex DNA containing a three-nucleotide 3' overhang that is observed to bind in the 3'-5' exonuclease active site. This editing complex shows duplex DNA bound at a right angle to the large cleft that contains the polymerase active site. This position for the duplex product of DNA synthesis implies that the DNA must make a large bend to put the primer terminus into the polymerase active site. (This research was supported by the American Cancer Society.)

The most significant advance in the previous year has been the preliminary determination of the structure of HIV reverse transcriptase (RT) complexed with a noncompetitive inhibitor, Nevirapine, discovered at Boehringer Ingelheim. Complexation with this inhibitor proved crucial to obtaining crystals that diffract to 3.0-Å resolution. The 3.5-Å resolution structure of the p66/p51 heterodimer is strikingly asymmetric: while the polymerase domain of p66 has a large cleft analogous to that of Klenow fragment, p51 shows no such cleft. One of the four polymerase subdomains has the same structure and conserved catalytic residues as Klenow fragment, suggesting that the catalytic subdomains of polymerases evolved from a common ancestor.

An A-form RNA-DNA hybrid can be model-built into the deep cleft that extends between the polymerase and RNase H active sites. Nevirapine binds in a pocket adjacent to the DNA and at the base of a thumb-like protrusion. Mutations in RT that are resistant to Nevirapine alter protein side chains that contact the inhibitor. They presumably lower the affinity of the enzyme for this component by changing the shape of the binding site. Mutations resistant to AZT (3'-azido-3'-deoxythymidine) and DDI (dideoxyinosine) are in residues that contact the template strand, making the structural interpretation of their phenotype unclear. (The reverse transcriptase studies were supported in part by the National Institutes of Health.)

Dr. Steitz is also Professor of Molecular Biophysics and Biochemistry and of Chemistry at Yale University.

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STRUCTURAL MOLECULAR BIOLOGY

DON C. WILEY, PH.D., *Investigator*

Class I histocompatibility glycoproteins carry short peptides derived from the cytoplasm of cells to the cell surface and present them to the surveillance of cytotoxic T lymphocytes (CTLs), which possess clonally distributed, hypervariable receptor molecules. CTLs specific for peptides derived from the organism's own proteins are eliminated or turned off by regulatory mechanisms. Thus if a CTL recognizes a histocompatibility glycoprotein-peptide complex on a cell surface, it is a signal that foreign peptides are being expressed in the cell such as would occur in a viral infection, and the CTL responds by killing the infected cell.

For their biological function, class I molecules must be able to bind peptides of many different sequences and to hold them very tightly. The binding must be essentially irreversible, so that peptide remains bound at the cell surface where the concentration of free peptide is near zero. In the past year, Dr. Wiley and his colleagues have determined and refined three class I structures to 2.1-, 2.8-, and 1.9-Å resolution and may have discovered the key to the unusually tight peptide binding.

The structure of HLA-B27 at 2.1-Å resolution (with Drs. Joan Gorga and Jack Strominger) demonstrated that the collection of endogenous (self) peptides bound to HLA (human leukocyte-associated antigens) in the crystal were predominately 9-mer with arginine at peptide position 2 (P2). Furthermore, the vast majority of the peptides bound with the same extended, kinked conformation, burying their amino and carboxyl termini in sites composed of residues conserved in all class I protein sequences.

The complex of a single-peptide antigen from influenza virus with the class I allele HLA-Aw68 was formed by *in vitro* reconstitution and its structure determined to 2.8 Å. The conformation of this single viral peptide bound to HLA-Aw68 is remarkably similar to that of the collection of endogenous peptides bound to HLA-B27, arguing that the mechanism of binding is general. The peptide is bound by its ends stretched out so that the sequence of most of the central residues can be read by the T cell receptor.

The structure of HLA-Aw68 complexed with the collection of endogenous peptides was refined to 1.9-Å resolution. Surprisingly, electron density for only the first three and last two peptide residues was visible. Peptide elution and sequence experiments indicate that peptides of various length, from 8-mer to 11-mer, bind tightly to HLA-Aw68, confirming the crystallographic interpretation that the center part of the peptide must bulge out of the site to varying degrees depending on peptide length.

These data together argue that the key to tight, essentially irreversible binding is fitting the amino and carboxyl termini into the conserved binding sites. Polymorphic residues in the binding sites determine which peptide sequences can fit their ends into the termini binding sites. Extensive hydrogen-bonding networks in both terminal sites would appear not only to hold the peptide but also to explain the peptide-dependent stability of the histocompatibility molecule's structure.

Dr. Wiley and his co-workers are currently refining a model of the human class II histocompatibility glycoprotein HLA-DR1 complexed with a collection of endogenous peptides. A crystal with a single influenza peptide has also been prepared, and diffraction data have been collected with synchrotron radiation, to at least 2.8-Å resolution. A complex of DR1 with the superantigen SEB is also being built at high

resolution. (The studies of DR1 are partially supported by the National Institutes of Health through grants to Drs. Strominger and Gorga.) These studies should offer insight into the function of class II molecules in regulating the production of immune sera.

A number of studies on influenza virus continue in collaboration with Dr. John Skehel in London. In the past year the resolution of the studies has been increased from 3.0 Å to ~2.2 Å by a combination of flash freezing and the use of phosphor image plates and the CHESS F1 synchrotron beam line. The laboratory has also begun characterizations of an inhibitor of virus-cell binding with 1,000-fold greater potency than sialic acid, the natural receptor. (Both the work on influenza virus and that of collaborators Drs. Jeremy Knowles and George Whitesides are supported by the National Institutes of Health.)

Studies on trypanosome variant-surface glycoproteins (VSGs) resulted this year in an analysis of two variant molecules with only 16% sequence homology but identical structure. A core of conserved amino acid residue type that appears to "hold" the structure together can be described. In some variants this core appears to be buried by carbohydrate residues rather than protein. The study establishes that antigenic variation in trypanosomes is caused by sequence variation and not by gross structural alterations. It also argues that the different classes of VSGs constitute a protein superfamily rapidly evolving within a single organism. (The trypanosome research is supported by the National Institutes of Health.)

The National Institutes of Health also supported work on influenza C virus, gp120 and gp160 of HIV-1, and a low-pH, fusion-active conformation of the influenza A virus hemagglutinin.

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INTERNATIONAL RESEARCH SCHOLARS

In recognition of the contributions of scientists outside of the United States to advances in biomedical science, the Institute initiated the International Research Scholars Program in 1991. This is a small and experimental effort that provides five-year grants to support the research of promising scientists working in areas of fundamental biomedical research related to the Institute's ongoing medical research programs.

Canada and Mexico were chosen as the eligible countries for awards in 1991. From those invited to compete, 24 were designated International Research Scholars (14 in Canada, 10 in Mexico), and grants were made to their institutions for support of their research. Grants also were awarded to the Mexican Academia de la Investigacion Cientifica and to the United States National Academy of Sciences for joint activities over four years to promote the exchange of scientific information and encourage cooperation between the scientific communities in each country, particularly in the life sciences.

The research reported here by the Scholars covers the work supported by HHMI funding during the first grant year (July 1, 1991–March 30, 1992), and the publications listed are those that have resulted from these awards.

Infection by animal rotaviruses is dependent on the interaction of the virions with sialic acid–containing structures on the surface of the target cell. In work reported by Carlos F. Arias, Ph.D. (National Autonomous University of Mexico, Cuernavaca) and his colleagues, the isolation and analysis of mutants that no longer depend on this interaction to be infective have revealed the existence of a novel site on the surface protein VP4 that is involved in the initial binding event, and probably penetration of the virion into the cell as well. In addition, a new cleavage site has been identified on VP4 that could play an important role in the trypsin enhancement of rotavirus infectivity.

Multicellular organisms have evolved communication mechanisms to ensure orderly development, neural function, and ongoing differentiative processes in the adult. A breakdown in this process of cell-cell communication can lead to a variety of diseases, including cancer. The laboratory of Alan Bernstein, Ph.D. (Mount Sinai Hospital, Toronto) is interested in applying the techniques of molecular genetics to dissect the molecular pathways that control these processes. Because cell-cell interactions take place in the intact animal, this group is taking various experimental approaches, involving analysis of existing mouse mutations and the generation of new mouse mutants carrying novel alterations in their germline. These approaches have led to the identification of several genes that encode a transmembrane receptor, a factor that specifically binds to this receptor, and novel proteins involved in regulating gene expression that are centrally involved in a variety of developmental processes and malignancies.

Several *Salmonella typhi* outer membrane protein (OMP) genes have been isolated by the laboratory of Edmundo Calva, Ph.D. (National Autonomous University of Mexico, Cuernavaca). The structure and regulation of *ompC*, which codes for a major OMP, has been studied intensively. Molecular characterization of these OMPs and their genes should allow better understanding of the human immune response during typhoid fever and of some genetic regulatory features in this bacterium. Genes specific for the two species of *Campylobacter* (*C. jejuni* and *C. coli*) that most frequently cause diarrhea in humans have been isolated and are being characterized in order to define these species at the molecular level.

Lactotropes comprise a heterogeneous population of prolactin-secreting cells in the vertebrate pituitary gland. By using cultured pituitary cells from adult male rats as a model system, Gabriel Cota, Ph.D. (National Polytechnic Institute, Mexico City) and his colleagues have shown the existence of two functional subsets of lactotropes that differ in basal secretory activity. In addition, they have provided electrophysiological evidence for a differential expression of voltage-gated ionic channels in the lactotrope subtypes. Their results indicate that the secretory behavior of an individual lactotrope is largely determined by the activity of Na^+ channels and high-threshold Ca^{2+} channels in its plasma membrane.

In the process of fertilization, cell signaling is a matter of life and death. Understanding the molecular mechanisms involved is the main goal of the laboratory of Alberto Darszon, Ph.D. (National Autonomous University of Mexico, Cuernavaca). Ionic fluxes are fundamental in sperm activation and in the induction of the acrosome reaction. The sperm must undergo this reaction to fertilize the egg. By combining planar bilayer and patch-clamp techniques with measurements of intracellular pH and calcium, this group has established the presence of

Ca^{2+}, K^+, and Cl^- channels in sea urchin sperm and explored their participation in chemotaxis and the acrosome reaction.

Many pathogenic bacteria are capable of exploiting host cell functions, including signal transduction and cytoskeletal rearrangements. These processes are under study in the laboratory of B. Brett Finlay, Ph.D. (University of British Columbia, Vancouver). Enteropathogenic *Escherichia coli* (EPEC) adhere to epithelial cells and cause a rearrangement of several cytoskeletal proteins. This adherence leads to disruption of transepithelial permeability. It also triggers host cell tyrosine kinase activity, resulting in tyrosine phosphorylation of a 90-kDa host cytoskeletal protein. Other invasive pathogens such as *Yersinia* and *Listeria* species also utilize host tyrosine kinases to mediate bacterial uptake into epithelial cells. Pirating of these functions appears to participate in disease production.

Initiation by human RNA polymerase II at the promoters of protein-coding genes requires several general factors. Interactions among these factors were characterized by Jack F. Greenblatt, Ph.D. (University of Toronto) and his colleagues, and factor requirements for recognition of a promoter were defined. The general factor TBP was found to be a target for several activator proteins: herpesvirus VP16, the human anti-oncogenic protein p53, and the human activator Sp1. Antitermination by the phage λ N protein in bacteria was used as a model system to study control of transcription termination. This system was reconstituted *in vitro* with seven pure proteins, and detailed mechanistic models for antitermination were developed.

The research of Sergio Grinstein, Ph.D. (University of Toronto) and his colleagues aims at understanding the mechanisms that regulate intracellular pH. Three separate systems were investigated: 1) Na^+/H^+ antiport was studied using phosphatase inhibitors. These agents phosphorylated the antiport and stimulated its exchange activity, suggesting that direct phosphorylation may be an important controlling mechanism. 2) A regulated H^+ conductance was also identified. Stimulated leukocytes depolarize and undergo a burst of metabolic acid generation. A sizable fraction of this acid is extruded via a pH-, voltage-, and kinase-sensitive conductance. 3) Proton pumps contribute to cytosolic pH regulation and to the establishment and maintenance of the acidic interior of phagosomes. Studies of ionic permeability indicated that the intrinsic pH sensitivity of vacuolar-type pumps is the main determinant of intraphagosomal pH.

P-glycoprotein (P-gp) is a membrane-bound drug efflux pump encoded by the *mdr* gene family, which is responsible for the onset of multidrug resistance (MDR) in tumor cells. The structure/function analysis of P-gp has been initiated by the laboratory of Philippe Gros, Ph.D. (McGill University, Montreal) in chimeric and mutant proteins, using full-length cDNA clones corresponding to the three members of the *mdr* gene family, and has shown that the membrane-associated domains are key structural determinants for substrate recognition. A new series of simple lipophilic cations has been identified as model substrates for P-gp. Functional complementation of the yeast *mdr* homologue *STE6* has been carried out successfully.

Bacterial mutants of the gene for peptidyl-tRNA hydrolase prevent phage λ vegetative growth. Wild-type and mutant alleles of the *pth* gene were cloned and sequenced by Gabriel Guarneros-Peña, Ph.D. (National Polytechnic Institute, Mexico City) and his colleagues, and the wild-type Pth protein was isolated and purified. Sequence analysis of two distinct phage mutations, termed *bar*, which overcome the host-cell Pth defect, revealed a nearly identical 16-bp DNA segment. Transcription through the wild-type *bar* region is required for λ exclusion, but in a plasmid vector, arrests protein synthesis and kills Pth-defective cells. A unifying model is proposed in which Bar RNA and Pth participate in termination of protein synthesis.

The laboratory of Luis R. Herrera-Estrella, Ph.D. (National Polytechnic Institute, Irapuato) is interested in the study of the molecular events that regulate carbon assimilation in plants. To understand these events, some of the DNA sequences and protein factors that mediate the tissue-specific and light-inducible expression of genes involved in photosynthesis have been identified. The role of molecular chaperones that have been implicated in the assembly of ribulose bisphosphate carboxylase have been studied using transgenic plants harboring antisense gene constructs for these molecular chaperone genes. The maize and rice genes encoding one of the key enzymes in carbon assimilation, the sucrose phosphate synthase, has been isolated.

One strategy that has been very effective in identifying mammalian genes required for pattern formation has been to use cross-species sequence conservation to clone related genes from vertebrates and invertebrates. A number of families of mammalian genes have been identified in this manner, based on their homology to *Drosophila* genes that control the process of segmentation. These families include the *Wnt* genes that are homologues of the *Drosophila wingless (wg)* gene, the *En* genes that are similar in structure to the *Drosophila engrailed (en)* gene, the *Pax* genes that contain a conserved paired

box domain that is found in the *Drosophila paired (prd)* and *gooseberry (gsb)* genes, and the *Gli* genes that have homology to the *Drosophila cubitus interruptus (Ci)* gene. Analysis of gene expression patterns and limited mutant analysis have indicated that these mammalian genes play important roles in pattern formation during gastrulation and organogenesis. In *Drosophila*, the *wg, en, prd, gsb,* and *Ci* genes have been shown to interact during segmentation in common genetic pathways. The expression patterns of the mouse homologues of these genes have shown that they may also interact similarly in mammals. Thus it is possible that not only the genes but also the genetic pathways have been conserved through evolution. Alexandra L. Joyner, Ph.D. (Mount Sinai Hospital, Toronto) and her colleagues have concentrated on determining the roles of the *En* genes in development and recently have begun to examine whether these genes interact with the *Wnt, Pax*, and *Gli* genes.

Paul M. Lizardi, Ph.D. (National Autonomous University of Mexico, Cuernavaca) and his colleagues are developing diagnostic assays based on RNA amplification. An enzymatic reaction is used to join two RNA molecules that contain sequences complementary to viral RNA. Joining occurs only if both probes are bound specifically to their target. The ligation reaction is mediated by a catalytic RNA known as a ribozyme. After joining, the resulting molecules are replicated by the enzyme Q-beta replicase, generating up to 100 million copies of the probe in 20 minutes. This novel method can be used for the rapid detection of infectious agents such as the AIDS virus.

The aim of the research of James D. McGhee, Ph.D. (University of Calgary, Alberta) is to understand how and why specific genes are expressed in some tissues, but not others, during an animal's development. Genes are modified in a test tube and then injected back into the host organism, a small free-living roundworm called *Caenorhabditis elegans*, to see if the modified genes still work correctly. Results to date suggest that a gene is actively turned on in the correct cells, at the same time that it is actively turned off in incorrect cells. These experiments are being extended to different roundworms to see how gene control mechanisms change during evolution.

The laboratory of Tim Mosmann, Ph.D. (University of Alberta, Edmonton) studies cytokines, small secreted proteins that mediate many of the functions of cells in the immune system. Two subsets of T lymphocytes, TH1 and TH2, have very different functions and cross-regulate each other via their unique cytokines. A new TH2-specific cytokine,

P600, has been found to induce the growth of macrophage-like cells from bone marrow precursors. These cells are able to process and present antigens to T cells, but it is not yet apparent how the functions of these cells relate to the known unique properties of TH2 lymphocytes.

Mechanisms involved in the virulence of *Entamoeba histolytica* are being investigated in the laboratory of Esther Orozco, Ph.D. (National Polytechnic Institute, Mexico City) by the identification of virulence-involved molecules and their expression through the switching of trophozoites from the nonpathogenic to pathogenic states. The 112-kDa adhesin demonstrates protease activity and is altered in *E. histolytica* virulence-deficient mutants. This adhesin and other virulence-involved molecules were detected only in pathogenic trophozoites. Genomic rearrangements, including gene amplification, correlate with the switching of trophozoites from nonpathogenic to pathogenic. The identification of a variable DNA fragment corroborated the high plasticity of the *E. histolytica* genome, which gives rise to genotypically and phenotypically different individuals from a single cell.

Many growth factors, and related hormones, regulate cell function through cell surface receptors with protein-tyrosine kinase activity. To understand the means by which such tyrosine kinases function, it is important to identify their immediate targets within the cell. The laboratory of Tony Pawson, Ph.D. (Mount Sinai Hospital, Toronto) has identified an element that appears to be a hallmark of tyrosine kinase targets, the Src homology 2 (SH2) domain. These domains of cytoplasmic signaling proteins bind to specific tyrosine-phosphorylated sites on activated growth factor receptors and thereby direct high-affinity interactions between receptors and their targets. SH2 domains also mediate a network of protein-protein interactions in the cytoplasm of cells stimulated with growth factors or transformed by oncogenic tyrosine kinases, which appear important to the control of signal transduction and the induction of cell proliferation.

The research in the laboratory of Lourival D. Possani, Ph.D. (National Autonomous University of Mexico, Cuernavaca) is focused on the study of the structure and function relationship of scorpion toxins, which are the peptides responsible for the lethal effects of scorpion stings. Among the recent accomplishments are 1) the isolation of two unknown Na^+ channel–blocking peptides from the venom of *Centruroides noxius* and the determination of their primary structure and some of their immunological characteristics; 2) the chemical and functional characterization of two newly purified

short-chain peptides (K$^+$ channel blockers) from the venom of the scorpion *Centruroides limpidus limpidus*; 3) the cloning of four genes that code for scorpion toxins of *C. noxius*; and 4) an analysis of the pancreatic secretagogue effect of scorpion toxins.

The research in the laboratory of Randy J. Read, Ph.D. (University of Alberta, Edmonton) is aimed at advancing the understanding of infectious diseases at the molecular level, using information from the three-dimensional structures of key proteins. There are two major aspects to this work. First, x-ray crystallography is employed to determine the structures of a number of proteins involved in the process of infection. Second, computational techniques are being developed to exploit such structural information in the rational design of new drugs for the treatment of infectious disease.

Over the past year, the laboratory of Ranulfo Romo, M.D., Ph.D. (National Autonomous University of Mexico, Mexico City) has investigated the representation of tactile signals in the cerebral cortex of awake monkeys. A first objective of this research was to define quantitatively the neural representation of moving tactile signals in the somatosensory cortex. It was found that neurons of the somatosensory cortex respond to tactile stimuli moving at speeds from 20 to 100 mm/s. Also, the direction of the moving tactile stimulus appears to be coded by a neuronal population distributed in the somatosensory cortex. These cortical neural signals should account for tactile performance.

The research of Janet Rossant, Ph.D. (Mount Sinai Hospital, Toronto) and her colleagues focuses on the early development of the mouse embryo. Studies at the time of gastrulation have indicated that one cell type, mesoderm, instructs the overlying ectoderm cells to make the tissue that will later form the brain. Retinoic acid, a vitamin A derivative, may be involved in anterior-posterior patterning of the brain at this time, as indicated through studies of transgenic mice designed to provide a readout of retinoic acid action in the embryo. Finally, new genes expressed at these early stages are being isolated by a "gene-trap" approach.

In one area of the cerebral cortex, visual information is transformed into information about the spatial distribution of objects in the environment. To understand this transformation, Jean-Pierre Roy, M.D. (McGill University, Montreal) and his colleagues study cells that respond when the subject moves in its environment. The laboratory examines how, in those cells, the response to certain characteristics of the visual input (for example, differential speed of motion of points in a visual display) could signal the relative depth of objects in the environment. The group's hypothesis stems from the observation that when one moves while examining an object, objects that are at different depths will appear to move at different speeds.

The entry of calcium into cells through voltage-gated calcium ion channels is responsible for a number of important physiological properties of different cell types. Much research has been directed toward understanding the diversity of calcium ion channels, and it has been proposed that distinct channels are selectively localized in nerve cells and that each type contributes uniquely to neuronal physiology. Utilizing molecular genetic techniques, the laboratory of Terry P. Snutch, Ph.D. (University of British Columbia, Vancouver) has determined the molecular basis for a large portion of the calcium ion channel heterogeneity that has been previously described. An unexpected finding is that a much larger number of channel isoforms can be defined at the molecular level than previously anticipated. The distinct isoforms are unequally distributed in the nervous system, and this suggests that they play different roles in the functioning of nerve cells.

The research interest of the laboratory of Lap-Chee Tsui, Ph.D. (Hospital for Sick Children, Toronto) concerns several general topics within the scope of the molecular biology of mammalian gene regulation and function. A major research activity centers around cystic fibrosis (CF). The identification of the gene and, subsequently, the different mutations causing the disease have provided the molecular basis for understanding the missing function in patients with CF, for DNA testing of individuals for carrier status, and for developing improved means of therapy. More recent work also shows that it is possible to correlate certain symptoms of the disease with the mutations carried by a patient. In another study the mutation causing a small and defective eye lens in a mutant mouse strain has been identified and serves as an excellent model for the study of human eye diseases. Yet another set of experiments has led to a new technique that promises to revolutionize the gene mapping field. The laboratory also has begun a significant effort on the characterization of the entire chromosome 7 as part of the international genome study, in order to facilitate the cloning of any disease genes that are located on it.

MOLECULAR BIOLOGY OF ROTAVIRUS INFECTION

CARLOS F. ARIAS, PH.D., *International Research Scholar*

Acute, infectious diarrhea is the commonest cause of morbidity and mortality among young children living in developing countries, accounting for as many as 1 billion illnesses and between 4 and 5 million deaths each year. Rotaviruses are the leading cause of severe gastroenteritis in children under three years of age, and there is considerable interest in developing effective vaccination strategies.

Fundamental to the construction of an effective vaccine is a basic understanding of the viral determinants that elicit protective immunity. In addition, a detailed knowledge of the molecular biology of the virus, especially of the initial events that lead to infection of the target cell, should contribute to the rational design of rotavirus vaccines and broaden the approaches to control of the viral infection.

The main interest of Dr. Arias and his colleagues is to study the molecular biology of rotaviruses, with particular emphasis on the interactions of the virion with the host cell early in the infection cycle. In addition, they are interested in the host immune response to rotavirus infection and the epidemiology of rotaviruses in Mexico.

Attachment and Penetration of Rotaviruses

The surface of the virus is formed by two proteins, VP4 and VP7. VP4 forms spikes that extend from the viral surface and is involved in a variety of viral functions, including virulence *in vivo*, agglutination of red blood cells (hemagglutination), and penetration of the virion into target epithelial cells. In contrast, the protein responsible for the initial attachment of rotavirus to the target cell remains controversial; both VP4 and VP7 have been proposed to play this role.

Hemagglutination and the attachment of animal rotaviruses to epithelial cells are mediated by sialic acid–containing compounds, since treatment of cells with sialidases inhibits hemagglutination and greatly reduces the binding of virus to the host cell surface. In addition, hemagglutination and the binding to target cells can be inhibited by incubation of the virus with a wide range of sialoglycoproteins, including glycophorin A, the protein in the red blood cell membrane that mediates the binding of the virus. Recently several different sialic acid–containing membrane components have been proposed to serve as binding sites for rotavirus particles in epithelial cells.

Virus mutants whose infectivity of epithelial cells is no longer inhibited by treatment of cells with si-alidases or incubation with glycophorin have been isolated by Dr. Arias and his co-workers. Paradoxically, these mutants are still able to hemagglutinate, and this hemagglutination is inhibited by glycophorin. Apparently glycophorin is able to bind to the mutant viruses but no longer prevents infection, suggesting that there is a second site on their surface, independent of the sialic acid–binding site, that is primarily responsible for interaction with the host cell.

Preliminary reassortment experiments indicate that this mutant phenotype segregates with the VP4 gene. The group has proposed as a working hypothesis that there are at least two sites on the VP4 protein of animal rotaviruses that interact with the cell surface during virus entry. In this model the first site mediates the initial interaction between the virus and the cell through a cell membrane sialic acid–containing structure, but this interaction alone does not lead to infection. After this initial event the second site mediates an interaction, probably with another cell membrane component, leading to penetration of the virion into the cell.

Previous reports indicate that rotaviruses bind to a wide variety of cell types. Productive infection, however, has only been observed to occur in a subset of the tested cells. This suggests that the first interaction is of a promiscuous nature, while the second is more specific and probably determines, at least in some cases, whether the cell is susceptible to infection.

The ability of the mutant viruses to infect in the presence of glycophorin or sialidase-treated cells could be explained if the mutation(s) had increased the affinity of the second interaction, such that the second site were able to interact effectively with the cell surface even in the absence of the first interaction. The infection of human rotaviruses is not dependent on sialic acids and is not inhibited by glycophorin, which would suggest that in this case the interaction of the human virus with the cell surface could be directly through a site similar to the second site identified in animal rotaviruses. Experiments are in progress to map the domains on VP4 responsible for these interactions.

The infectivity of rotaviruses is increased, and is probably dependent on, trypsin treatment of the virus. Previously, Dr. Arias and his co-workers identified two specific cleavage sites on VP4, at conserved arginines 241 and 247, and proposed that cleavage at these sites enhances infectivity. Recently

they have found that cleavage also occurs at arginine 231, which is conserved in all rotavirus strains analyzed so far. This may be the site in VP4 that is most susceptible to trypsin cleavage.

In addition, they have shown that digestion of the virus with proteolytic enzymes other than trypsin—enzymes that generate single cleavages at positions 241 or 245 in VP4—does not activate viral infectivity. After these treatments, however, the virion infectivity can still be activated by trypsin. The results suggest that the hitherto unidentified cleavage site at arginine 231 might play a key role in the activation of the virus infectivity. Dr. Arias and his colleagues are currently interested in identifying the cleavage(s) directly responsible for the enhancement of viral infectivity and in elucidating the mechanism through which the cleaved VP4 protein mediates penetration of the virion into the cell.

Immune Response to Rotavirus Infection

So far six different serotypes of human rotaviruses have been identified, four of which appear to account for the great majority of isolates worldwide. Dr. Arias and his co-workers have been investigating the relationship between the serotype of the infecting rotavirus and the specificity of the immune response in infected children. In apparently primary infections, they have shown that rotaviruses are able to induce a neutralizing immune response of a heterotypic nature—i.e., directed to more than one serotype—and that this antibody response is elicited preferentially by viruses of serotypes 2, 3, and 4. The group's current interest is to determine the molecular basis of this heterotypic cross-reactive immune response, which appears to be an intrinsic property of certain rotaviruses. An understanding of this immune response could be very helpful in the design of vaccines that would protect against a variety of rotavirus serotypes.

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MOLECULAR GENETICS OF CELL SIGNALING IN MAMMALS

Alan Bernstein, Ph.D., *International Research Scholar*

Cells of multicellular organisms need mechanisms for the transmission and reception of extracellular signals and for transducing them across the plasma membrane to activate intracellular signaling pathways that lead to an appropriate proliferative or developmental response. The hematopoietic system provides a particularly good example of the critical role these signaling processes play in regulating the orderly production of blood cells. All the cells in the blood and in lymphoid organs arise as a result of the ongoing proliferative and developmental divisions of a hierarchy of pluripotent stem cells that reside in the adult bone marrow. Molecular analysis of the diseases that result in either under- or overproduction of mature blood cells has demonstrated the tight controls that govern stem cell function and has helped in the dissection of the molecular pathways that regulate these cells.

Control of Hematopoiesis

Dr. Bernstein's laboratory is attempting to understand the molecular and cellular basis of normal blood cell production by identifying cellular genes that play critical roles in this process. Several experimental approaches are being taken by the group to understand this complex system. They are analyzing the genes and their protein products that are important to the evolution of the multistage erythroleukemias induced by various strains of Friend leukemia virus. The molecular analysis of existing mouse developmental mutations that affect normal hematopoiesis provides a second approach to the identification of genes that are critical to cellular interactions in the blood-forming system. And finally, the introduction of either loss- or gain-of-function mutations into the mouse germline of genes normally expressed in hematopoietic cells

provides a genetic approach to dissection of the molecular components of signaling pathways that regulate developmental processes in this system.

Molecular Basis of Friend Erythroleukemia

Cancer in both humans and experimental animals is a multistage process involving the activation of dominant-acting oncogenes and the inactivation of tumor-suppressor genes. The complex nature of this process raises many questions concerning the interactions among these genes and their protein products that lead to unregulated cell growth. The erythroleukemias induced by the various strains of the murine retrovirus Friend leukemia virus have provided an excellent experimental system in which to study the multistage nature of cancer.

By studying the molecular changes in leukemic clones that emerge late after viral infection, it became evident that inactivation of the p53 gene is a frequent and probably obligate step in the evolution of Friend leukemia. The conclusion that p53 is a tumor-suppressor gene has been confirmed and extended by recent experiments demonstrating mutation and allelic loss in a broad spectrum and high proportion of human cancers and by the finding that wild-type p53 can inhibit the transforming ability of mutant p53.

In addition to inactivation of the p53 gene, activation of one of two novel members of the *ets* gene family of DNA-binding transcriptional activators occurs during the evolution of Friend leukemia. The *ets* gene *Spi-1* (spleen focus-forming virus preferred integration site 1) is activated by proviral insertion in 95% of leukemic clones induced by Friend, whereas another novel *ets* gene, *Fli-1*, is activated by insertion of the replication-competent Friend murine leukemia virus. The strict specificity of integration sites suggests that *Fli-1* and *Spi-1* are functionally distinct, encoding proteins that transactivate a distinct set of genes downstream in the leukemogenic pathway. Consistent with this possibility, Dr. Bernstein and his colleagues have shown that DNA-binding ETS domains of *Fli-1* and *Spi-1* recognize distinct DNA sequences and transactivate distinct transcriptional regulatory regions in *in vitro* expression studies. Experiments are under way to generate animals harboring mutations in either the *Fli-1* or *Spi-1* genes, to determine their phenotype and assess their susceptibility to erythroleukemia induction.

The *white-spotting* and *Steel* Loci

Mutations in mice at either the dominant *white-spotting* (*W*) or *Steel* (*Sl*) loci can lead to coat color defects, severe macrocytic anemia, and sterility. Consistent with the intrinsic and extrinsic nature of the *W* and *Sl* phenotypes, respectively, *W* encodes the Kit receptor tyrosine kinase and *Sl* encodes its ligand. As expected from earlier biological analysis of *W* and *Sl* mutants, c-*kit* is expressed in early hematopoietic progenitor/stem cells—melanoblasts, melanocytes, and primordial germ cells. *Sl* is expressed in cells that immediately surround those expressing c-*kit*, including fibroblasts, Sertoli cells, and granulosa cells. Thus the Kit signaling pathway appears to be activated by cell-cell contact, a conclusion supported by the observation that the Sl^d mouse mutant, which fails to make the membrane-bound form of the Steel protein, has a mutant *Sl* phenotype.

This close apposition of cells expressing *W* and *Sl* is found not only in the three cell lineages affected by *W* or *Sl* mutations but also, for example, in the brain, where c-*kit* and *Sl* are found at very high levels in the hippocampus and cerebellum. These observations suggest that *W* and *Sl* might play a critical role in neural function—in neural development, axonal guidance, and/or higher neural functions. Mice homozygous for severe *Sl* or *W* point mutations display no obvious abnormality or deficit of c-*kit*–expressing neuronal cells in the brain, suggesting that this signaling pathway plays a postmitotic role in the central nervous system.

Together these approaches to cell signaling should contribute to emerging concepts concerning the molecular basis of cell-cell communication during normal embryological development, hematopoiesis, neural function, and malignancy.

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MOLECULAR BIOLOGY OF TWO ENTEROPATHOGENIC BACTERIA

Edmundo Calva, Ph.D., *International Research Scholar*

Typhoid fever (TF) in humans is the clinical manifestation of a systemic infection by *Salmonella typhi*, a gram-negative enterobacterium, which is usually ingested via contaminated food or water. TF is estimated to affect annually more than 12 million persons worldwide, and the case fatality rate is 1%. The process of infection entails a series of bacteria-host interactions that are amenable to the basic study of a variety of biological phenomena. The mechanisms by which *Salmonella* reaches the intestinal tract, adheres to intestinal cells, resists phagocytosis, and multiplies within macrophages are largely unknown, although rapid progress has been achieved in the past few years regarding the molecular mechanisms involved in the invasion of epithelial cells by bacterial pathogens. Research in TF thus offers the opportunity to explore many aspects of bacterial molecular genetics, pathogenesis, and host immune response. The information obtained could provide relevant insights for the design of improved diagnostic tests and vaccines for TF, as well as on the biological processes involved in other bacterial infections.

Diarrheal diseases are considered the second leading cause of global deaths from illness, with an estimated number of 5 million during 1991. The fatalities are second only to those from cardiovascular disease and exceed those from cancer, pneumonia, tuberculosis, or AIDS. *Campylobacter jejuni*, a gram-negative, vibroid bacterium, is one of the major causal agents of diarrhea worldwide. Knowledge is scarce on the genome structure and function of this organism and, in the same context as described above for *S. typhi*, on its pathogenic mechanisms. Therefore studies in this field also offer opportunities for the probing of interesting biological phenomena, with the added benefit of providing knowledge potentially useful for health interventions.

Salmonella typhi Outer Membrane Proteins

The outer membrane proteins (OMPs) of a variety of gram-negative bacteria have been shown to be immunogenic in various host systems, including *S. typhi* OMPs in humans. In this respect, Dr. Calva and his group, in collaboration with Dr. Guillermo Ruíz-Palacios and his colleagues (National Institute for Nutrition, Mexico City) have shown the usefulness of OMP preparations for the rapid diagnosis of TF in patients from different parts of the world.

Dr. Calva and his colleagues started their molecular studies on *S. typhi* by isolating OMP genes with sequence similarity to three *Escherichia coli* OMP genes: *ompC*, *ompF*, and *phoE*. They determined that the *S. typhi ompC* gene codes for a major OMP whose expression is influenced by medium osmolarity differently than that of its extensively studied counterpart in *E. coli*. Main variable regions within the *S. typhi ompC* gene were defined, most of which appear to code for segments of the OmpC protein that are exposed to the cellular exterior, as assessed by comparative studies with other OMP sequences. Topological studies are being performed by constructing chimeric genes, coding for OmpC proteins with a foreign epitope inserted into a particular region, and then determining whether this epitope is exposed to the cell surface. Their current research also includes characterization of the genetic factors that influence *ompC* expression, such as the *envZ* and *ompR* genes. These factors, especially those involved in the response to osmotic stress, could well form part of a global genetic circuitry that controls a variety of genes, including some involved in pathogenesis.

An *ompF*-like gene has been isolated and is under study in the laboratory. Unexpected features distinguish it from *E. coli ompF*, such as coding for a protein of unusually different size, with another pattern of expression. The *S. typhi phoE* gene is being characterized. The search for and examination of other OMP genes with sequence similarities to *ompC* or *ompF* are current undertakings of Dr. Calva and his group.

In terms of molecular phylogeny, the *ompC* gene is being used as a probe for genetic variation within the *Salmonella* genus by oligonucleotide hybridization and PCR (polymerase chain reaction) technology. Use is made of nucleotide sequence information corresponding to variable and conserved segments. In this manner the usefulness of *ompC* as a DNA probe for diagnostics is being assessed.

Molecular Taxonomy of *Campylobacter*

Campylobacter jejuni and *C. coli* are the two species of *Campylobacter* most frequently associated with human enteritis. Their genomes share extensive homology, although variable and conserved DNA sequences have not been comprehensively defined. The two species are differentiated mainly on the basis of a single biochemical test, and it is not clear whether they have different modes of pathogenesis. Dr. Calva's group has isolated two DNA probes for *Campylobacter*. One is specific for *C. jejuni* and *C. coli*, and the other allows distinction between most isolates of these two species, by hybridizing preferentially with *C. jejuni*. Aside from their potential role in molecular epidemiology, the genes contained in both probes are being analyzed, since this presents a favorable opportunity to learn more about the uniqueness of these organisms.

The molecular characterization of a *Campylobacter* cholera-like enterotoxin (CJT) has been undertaken in collaboration with Dr. Ruíz-Palacios and his colleagues, who were the first to describe the presence of CJT in some *C. jejuni* clinical isolates. These studies make possible future research on the function of CJT in the bacterial cell, and more precise classification of the clinical isolates.

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FUNCTIONAL PROPERTIES OF PROLACTIN-SECRETING CELLS

Gabriel Cota, Ph.D., *International Research Scholar*

Dr. Cota's research is aimed at understanding the cellular mechanisms involved in the control of prolactin secretion in the vertebrate pituitary gland. Prolactin is a versatile hormone that participates in the regulation of a variety of physiological processes, including lactation. It is produced by pituitary cells called lactotropes or mammotropes. Until recently lactotropes were commonly thought to comprise a homogeneous cell population in the normal gland. However, studies performed on cultured pituitary cells indicate the existence of distinct subsets of prolactin secretors that differ in basal secretory activity or responsiveness to extracellular regulatory factors. A major goal of Dr. Cota's research is to define the intrinsic properties of lactotrope subpopulations that determine their different secretory behavior.

Lactotrope Subtypes

Dr. Cota's group has explored the differences in the basal rate of prolactin secretion among individual lactotropes in primary cell cultures obtained from pituitaries of adult male rats. Prolactin release was visualized and quantified at the single-cell level by using the reverse hemolytic plaque assay. In this immunological technique, cells releasing the appropriate hormone induce lysis of indicator erythrocytes, and the size of the zone of hemolysis, or plaque, around an endocrine cell provides an index of its secretory activity. Two subtypes of prolactin secretors were identified: small-plaque (SP) lactotropes that released small amounts of prolactin per unit time under basal conditions and comprised about 6% of all pituitary cells, and large-plaque (LP) lactotropes that secreted prolactin at higher basal rates and accounted for about 14% of the pituitary cell population.

Ca^{2+} Channel Activity and Lactotrope Heterogeneity

Like other endocrine cells, lactotropes are electrically excitable and exhibit spontaneous depolarizations of membrane potential. Basal prolactin secretion is thought to be sustained by Ca^{2+} influx through voltage-gated Ca^{2+} channels in the plasma membrane, which transiently open during spontaneous action potentials. Dr. Cota's laboratory has analyzed the Ca^{2+} channel activity of identified prolactin secretors using electrophysiological methods.

Whole-cell recordings of ionic currents with the patch-clamp technique revealed the presence of two classes of voltage-gated Ca^{2+} channels in the plasma membrane of each lactotrope examined: low-threshold and high-threshold channels. The activity of low-threshold channels did not significantly differ between SP and LP lactotropes. By contrast, the surface density of high-threshold channels was markedly larger in LP secretors. Plaque assays were again used to demonstrate that blocking the high-threshold Ca^{2+} channels with pharmacological agents selectively inhibits prolactin secretion from LP lactotropes. In fact, many of these cells behaved functionally as SP secretors in the presence of the channel blockers. These results indicate that high-threshold Ca^{2+} channels are differentially expressed in lactotrope subtypes, and strongly suggest that Ca^{2+} entry through such ionic channels is a crucial determinant of the amount of prolactin released from a cell.

Functional Relevance of Na^+ Channels in Lactotropes

During the past year Dr. Cota and his colleagues have succeeded in characterizing an additional difference in ion channel activity between the two lactotrope subtypes. In patch-clamp experiments, membrane depolarization was found to induce whole-cell Na^+ currents in LP secretors that were stronger than in SP cells. Such differences in Na^+ current amplitude were not related to cell-to-cell variations in the kinetic properties of the ionic currents and persisted after normalizing current amplitude by cell capacitance, a procedure that eliminates membrane area as a variable. Thus, like high-threshold Ca^{2+} channels, Na^+ channels are not uniformly expressed among SP and LP lactotropes.

Na^+ channels should be functionally important for the secretory activity of lactotropes, as they favor the triggering of action potentials and thereby promote the opening of high-threshold Ca^{2+} channels. Consistent with this view, it was found that tetrodotoxin, a potent blocker of Na^+ channels, drastically decreases the total amount of prolactin secreted from the cultured pituitary cells. Furthermore, population analysis of prolactin plaque sizes suggested that this inhibitory action of tetrodotoxin can be explained by the preferential suppression of LP lactotropes.

Research in Dr. Cota's laboratory is now focused

on the postnatal development of the lactotrope population. In addition, studies are in progress to identify extracellular factors that regulate ion channel expression in pituitary cells.

Dr. Cota is Professor of Physiology, Biophysics, and Neurosciences at the Center for Research and

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IONIC FLUXES IN THE ACROSOME REACTION OF SEA URCHIN SPERM

Alberto Darszon, Ph.D., *International Research Scholar*

Dr. Darszon and his colleagues have continued their efforts to understand at the molecular level the relationship and regulation of the ionic fluxes that are deeply involved in the sea urchin sperm acrosome reaction (AR).

In sea urchin sperm, the AR is triggered by a fucose-sulfate–rich polymer, FSG, contained in the outer investment of the egg, the jelly. FSG induces the influx of Ca^{2+} and Na^+ and the efflux of K^+ and H^+, leading to increases in intracellular concentration of Ca^{2+} ($[Ca^{2+}]_i$) and intracellular pH (pH_i), a K^+-dependent transient hyperpolarization that may involve K^+ channels (in *Lytechinus pictus* sperm) and a Ca^{2+}-dependent depolarization.

Desensitization of the Egg Jelly Receptor

In *Strongylocentrotus purpuratus* sperm suspended in 2 mM Ca^{2+} artificial sea water (ASW), egg jelly and FSG induce a small and transient increase in $[Ca^{2+}]_i$ without producing the AR. Even after Ca^{2+} restoration to 10 mM, its normal concentration in ASW, sperm are unable to attain the AR or to respond to a new addition of FSG after being washed and resuspended in ASW. By varying the time between the addition of FSG and the restoration of Ca^{2+} to 10 mM, it is possible to determine that the rate of Ca^{2+} influx inactivation is accelerated as the concentration of external Ca^{2+} increases. The AR after Ca^{2+} restoration also decreases as external Ca^{2+} increases. However, raising $[Ca^{2+}]_i$ with ionomycin does not lead to the refractory state in sea urchin spermatozoa. High external pH was used to trigger Ca^{2+} influx and the AR without activating the egg jelly receptor. External pH was unable to induce a refractory state in sea urchin sperm suspended in 2 mM Ca^{2+} ASW, even though it increased $[Ca^{2+}]_i$. This indicates that FSG or egg jelly is required to achieve the refractory state in sea urchin sperm and that desensitization of the egg jelly receptor is probably involved in this process.

Membrane Potential and K^+ Channels in the Acrosome Reaction

To investigate the role of the membrane potential changes during the AR, *L. pictus* sea urchin sperm were artificially hyperpolarized with valinomycin in K^+-free ASW (0KASW). This condition raised pH_i, caused a small increase in $^{45}Ca^{2+}$, and induced some AR. When the cells were depolarized 40–60 s after the induced hyperpolarization, the pH_i decreased and there was a significant increase in $^{45}Ca^{2+}$ uptake, $[Ca^{2+}]_i$, and the AR. The waiting time was required to allow the pH_i change necessary for the AR to occur.

Planar bilayers into which *L. pictus* plasma membranes were incorporated by fusion displayed single cation-selective channels of 85 pS. Although the selectivity sequence of this channel for cations needs to be determined, its single-channel conductance is close to that found for one of the K^+ channels of *S. purpuratus* plasma membranes.

Three conclusions can be drawn from these results. 1) Part of the K^+-induced depolarization seen in *L. pictus* sea urchin sperm suspended in 0KASW is due to Ca^{2+} influx through a voltage-dependent Ca^{2+} channel. 2) The K^+-dependent hyperpolarization in sperm of this species is important to trigger the AR and may be necessary to *a*) remove inactivation from voltage-dependent Ca^{2+} channels so that a subsequent depolarization could open them and/or *b*) to increase pH_i stimulating a Na^+-H^+ exchange. This alkalinization is necessary for the AR and may participate in the regulation of a Ca^{2+} transport system activated in this process. 3) The bilayer experi-

ments, together with previous patch-clamp results, indicate the presence of K$^+$ channels in the plasma membrane of *L. pictus* sea urchin sperm.

A cAMP-Dependent Protein Kinase Associated with the Sea Urchin Sperm Plasma Membrane

Cell motility and the AR are accompanied by an increased cyclic nucleotide metabolism and protein phosphorylation. Early studies with isolated plasma membranes from *L. pictus* and *Arabacia punctulata* invoked a cGMP-dependent protein kinase in the GTP and egg peptide (speract- and resact-) dependent phosphorylation of several membrane polypeptides. However, the nature of the interaction between the kinase and the membrane was not explored.

Isolated flagellar plasma membranes from *S. purpuratus* sea urchin sperm incubated with [γ-^{32}P]ATP in the presence of 1 μM cAMP showed an increased phosphorylation in several polypeptides. Half-maximal response was seen at 0.6 μM cAMP, while much higher cGMP concentrations (100 μM) were required to detect a similar protein phosphorylation. Most (80%) of the cAMP-stimulated protein kinase was resistant to extraction by 10 mM EGTA and sonication. In contrast, all the activity was recovered in a detergent-solubilized fraction.

Membranes pretreated with 200 μM cAMP, ultracentrifuged, and resuspended in buffer solution did not undergo cAMP-stimulated phosphorylation in their polypeptides. Therefore the cAMP-dependent protein kinase appears to be bound to the flagellar plasma membranes isolated from *S. purpuratus* sea urchin sperm via its regulatory subunit. This kinase has also been detected in isolated sperm head membranes and may participate in motility and the AR.

Swollen Sea Urchin Sperm: A New Model to Study the Role of Ionic Channels in the Response to Egg Factors

Sea urchin sperm are very tiny cells; the head diameter is ~2 μm. This has precluded a careful characterization of their electrophysiological properties that would shed light on the molecular mechanisms that determine the fascinating egg-induced behavioral changes. Recently, in collaboration with Drs. Donner Babcock and Martha Bosma (University of Washington, Seattle), it was found that one can hypotonically swell sea urchin sperm. The swollen cells are spherical (~4 μm in diameter), immotile, and metabolically active; and they can respond to speract as normal cells do, with changes in membrane potential, [Ca^{2+}]$_i$, and pH$_i$. Swollen sperm can be reproducibly patch-clamped, and single channels can be recorded. Speract at pM concentrations activates a small cation channel.

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Article

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EXPLOITATION OF HOST SIGNAL TRANSDUCTION PATHWAYS BY PATHOGENIC BACTERIA

B. Brett Finlay, Ph.D., *International Research Scholar*

Many pathogenic bacteria that cause disease establish intimate relationships with host cells. These range from adherence to tissue cells to entry into host cells and intracellular replication (also known as intracellular parasitism). It is becoming increasingly apparent that pathogenic microbes are able to exploit several host cell functions, including signal transduction pathways and cytoskeletal functions, and that such exploitations are required for the establishment of disease.

Enteropathogenic *Escherichia coli* (EPEC) Triggers Cytoskeletal Rearrangement and Host Protein-Tyrosine Phosphorylation

EPEC is a major cause of pediatric diarrhea, especially in developing nations. However, the mecha-

nisms by which this organism causes disease remain undefined. When EPEC adheres to epithelial cells, it causes the loss of microvilli. Accompanying this loss is the accumulation of host actin beneath the adherent organism, forming a pedestal upon which the organism rests. Several other epithelial cytoskeletal proteins were identified that also accumulate beneath this organism. These include the actin-crosslinking protein α-actinin and the two proteins talin and ezrin, which are involved in linking the cytoskeleton to membrane receptors. However, the cellular distribution of tropomyosin, tubulin, vinculin, and the intermediate filament proteins keratin and vimentin are not affected by EPEC.

Salmonella typhimurium is another diarrhea-causing organism that rearranges host cytoskeletal proteins. However, the rearrangements caused by the two organisms appear different. Although both alter actin, the actin beneath EPEC is tightly localized and outlines the adherent organism. In contrast, actin rearrangement triggered by *S. typhimurium* appears stringier, but still surrounds the invading organism. *S. typhimurium* triggers rearrangement of α-actinin, talin, and ezrin. In contrast to EPEC, *S. typhimurium* also causes accumulation of tubulin and tropomyosin. It appears that the EPEC-induced rearrangements are stable and form a structure on which the adherent organism remains, while *S. typhimurium* transiently induces changes that are associated with actin-myosin–mediated uptake of the organism into the epithelial cell.

Given EPEC's role in diarrhea, the effect of EPEC on the impermeability of polarized epithelial cells was also examined. EPEC causes a significant decrease in transepithelial electrical resistance of polarized Madin-Darby canine kidney (MDCK) and human intestinal Caco-2 epithelial monolayers. This decrease occurs ∼10 h after bacterial addition and appears to be mediated by intracellular organisms. Mutations in EPEC that affect actin accumulation and bacterial invasion (*cfm* and *eae*) do not cause the decrease in transepithelial resistance. These mutants also do not cause diarrhea in human volunteers. Collectively this information suggests that an increase in transepithelial permeability may be involved in EPEC-mediated diarrhea.

The cytoskeletal rearrangement that accumulates beneath adherent EPEC stains brightly with monoclonal antibodies directed against phosphotyrosine. When HeLa cells are infected with EPEC, a 90-kDa HeLa cell protein becomes heavily tyrosine phosphorylated. This tyrosine-phosphorylated protein is associated with the cytoskeleton and membrane. Tyrosine protein kinase inhibitors block tyrosine phosphorylation of the 90-kDa protein. These inhibitors also block EPEC uptake into HeLa cells. Additionally, a mutant strain of EPEC, *cfm*, which adheres normally but cannot cause actin accumulation, does not trigger tyrosine phosphorylation of the 90-kDa protein. Thus it appears that the EPEC *cfm* locus is involved in triggering host cell tyrosine kinase activity, while the product of *eae* nucleates and organizes actin into an ordered structure beneath the bacterium. When mixed in equal amounts, *cfm* and *eae* mutants are capable of complementing cytoskeletal rearrangement and invasion of the *cfm* mutants but not the *eae* mutants.

Host Tyrosine Kinase Activity Is Necessary for *Yersinia* and *Listeria* Uptake Into Epithelial Cells

Several pathogenic bacteria have the capacity to be internalized into nonphagocytic cells, such as epithelial cells. *Yersinia* species have several products that are involved in invasion. The best characterized is invasin, a bacterial protein that interacts with β_1-integrins to mediate uptake. Several tyrosine kinase inhibitors, including staurosporine, genistein, and a tyrphostin, block invasin-mediated uptake into epithelial cells without affecting bacterial adherence or viability. The effect of staurosporine on invasion is rapidly reversible, with bacteria being internalized within 2 min after drug removal. These results indicate that invasin-mediated internalization triggers host tyrosine kinase activity, presumably through a β_1-integrin linkage with the bacterium.

Gram-positive *Listeria monocytogenes* is another organism that is capable of entering into nonphagocytic cells. Its uptake into cultured epithelial cells is also significantly blocked by host tyrosine kinase inhibitors. In contrast, invasion of *S. typhimurium* is not affected by these inhibitors.

Collectively it appears that pathogenic bacteria have the capacity to adhere to host cell surfaces through defined receptors and then to pirate host cell events, such as signal transduction and cytoskeletal rearrangement, for bacterial benefit, and that these actions potentiate disease.

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FUNDAMENTAL MECHANISMS IN TRANSCRIPTIONAL REGULATION

JACK F. GREENBLATT, PH.D., *International Research Scholar*

Initiation and termination of transcription are the principal fundamental processes whose regulation leads to control of gene expression in all cells. Dr. Greenblatt's objective is to characterize the enzymology of these processes and to understand how particular regulatory mechanisms act upon them. Because basic processes are often remarkably conserved among living organisms, these studies are carried out in both prokaryotes and eukaryotes. The study of transcription termination focuses on the bacterium *Escherichia coli* and its temperate bacteriophage λ, while studies on transcriptional initiation are performed with human cells.

Transcriptional Antitermination by the N Protein of Phage λ

Each early operon of phage λ contains multiple transcriptional terminators. The phage N protein prevents termination at all these terminators and thereby allows the expression of genes that are essential for the growth of the phage. Each early λ operon is a target for N action because it contains an N utilization site (*nut* site). Antitermination by N is assisted by the four host *E. coli* factors NusA, NusB, NusG, and ribosomal protein S10.

A major recent objective of the Greenblatt laboratory was the reconstitution of antitermination by N *in vitro* in reactions containing only seven purified proteins. This objective was accomplished with the identification and purification of NusG, a protein encoded by an *E. coli* gene in which the *nusG4* mutation suppresses the effect of the *nusA1* mutation on antitermination by N. NusG was shown to interact directly with RNA polymerase and to travel together with N, NusA, NusB, and S10 on the surface of RNA polymerase in N-modified transcription complexes. It was also shown that the *nut* site is made of RNA and is assembled together with N and the Nus factors into a ribonucleoprotein particle on the surface of RNA polymerase. Antitermination at a terminator located just downstream from a *nut* site requires only an unstable complex containing N and NusA, but processive antitermination that persists for kilobases of DNA additionally requires NusB, NusG, and S10.

Two of the host factors, NusB and S10, were shown to form a heterodimer that associates with RNA polymerase through ribosomal protein S10. As well, the NusB-S10 complex was found to bind directly to the *boxA* antiterminator element in *E. coli* ribosomal RNA (*rrn*) operons. This *boxA* element found in *rrn* operons is closely related to the *boxA* component of a λ *nut* site. The *nut* site *boxA* element behaves as a mutated evolutionary descendant of the *rrn* operon *boxA* element, which cannot, by itself, bind NusB and S10 in the absence of a *boxB* element that, in turn, cooperatively binds N and NusA. In collaboration with the laboratory of Dr. Catherine Squires (Columbia University), Dr. Greenblatt's laboratory has established an *in vitro* system to study antitermination in the ribosomal RNA system and has shown that antitermination in this system also depends on NusB.

NusG affinity chromatography was used to show that NusG interacts with the termination factor Rho. In the absence of N, this interaction facilitates termination by Rho *in vitro*, but it is also important for antitermination by N. Dr. Greenblatt has proposed that the NusG molecule in an N-modified transcription complex sequesters Rho factor as it approaches the transcription bubble before Rho can interact with RNA polymerase and terminate transcription.

Initiation of Transcription by RNA Polymerase II

The general initiation factors assemble in an ordered pathway with RNA polymerase II at promoters for protein-coding genes. The TATA box usually present in such promoters is recognized by TBP, the DNA-binding subunit of the general factor TFIID. Using TBP affinity chromatography, Dr. Greenblatt's laboratory showed that TBP interacts with the human general factor TFIIA and that TFIIA has three subunits: A35, A21, and A12. Site-specific protein-DNA crosslinking was used to establish the alignments of TBP, A35, and A21 along promoter DNA.

The general factor TFIIF has two subunits, RAP30 and RAP74. Dr. Greenblatt and his colleagues had previously cloned human cDNAs encoding RAP30, and this year, in collaboration with Dr. Zachary Burton (Michigan State University), they cloned human cDNAs encoding RAP74. RAP30 behaves in several ways like a bacterial σ factor: it binds to *E. coli* RNA polymerase in competition with σ^{70}; it prevents nonspecific binding of RNA polymerase II to DNA; and it recruits RNA polymerase II to a preformed preinitiation complex containing TFIID and TFIIB. Recombinant TBP, TFIIB, and RAP30 were found to be sufficient for promoter recognition by RNA polymerase II.

Activation of Transcription by RNA Polymerase II

Activator proteins usually have two critical domains. One binds to DNA and the other interacts with the transcription apparatus. In collaboration with the laboratory of Dr. C. James Ingles, Dr. Greenblatt's group showed previously that the acidic activation domain of the Herpes simplex virus activator VP16 interacts with TBP. Similarly, the acidic activation domain of the human anti-oncogenic protein p53 binds to TBP, and this interaction is prevented by at least some oncogenic mutations in p53. Recent data indicate that even Sp1, a ubiquitous human activator whose activation domains are glutamine-rich rather than acidic, binds to TBP, suggesting that TBP may be a universal target for activator proteins. However, other recent data in the Greenblatt laboratory indicate that activation domains may also contact the general factor TFIIH and a 100-kDa protein whose role in transcription is unknown. The mechanism that activates transcriptional initiation is clearly complex and is currently the subject of intensive investigation.

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REGULATION OF ION TRANSPORT AND INTRACELLULAR pH

SERGIO GRINSTEIN, PH.D., *International Research Scholar*

Coordination of the activity of the myriad cellular enzymes requires accurate and continuous regulation of the intracellular pH. The cytosolic compartment tends to become acidic as a result of metabolic generation of protons (H^+). In addition, the transmembrane potential drives the passive electrophoretic accumulation of H^+ equivalents inside the cell. These processes must be effectively counteracted in order to maintain pH homeostasis. Dr. Grinstein's laboratory is studying the mechanisms responsible for the regulation of intracellular pH.

Na⁺/H⁺ Antiports

A family of electroneutral exchangers utilize the inward Na^+ gradient to drive the extrusion of H^+ across the plasma membrane. These so-called Na^+/H^+ exchangers, or antiports, play a central role in the maintenance of intracellular pH, but have also been implicated in the control of cellular volume and in the stimulatory effects of some hormones and growth factors.

When activated by cell shrinkage or growth promoters, the internal pH dependence of the exchange reaction undergoes an alkaline shift. The molecular basis of this alteration in kinetic behavior is poorly understood. To assess the role of protein phosphorylation, lymphoid cells were exposed to okadaic acid, a selective inhibitor of phosphatases 1 and 2A. In otherwise untreated cells, okadaic acid promoted a rapid activation of the antiport, suggesting the presence of constitutively active kinases. Indeed, a sizable increase in protein phosphorylation was detectable under comparable conditions.

The enhancement of antiport activity by okadaic acid was not additive with the osmotically induced stimulation, suggesting common pathways. However, the two effects were differentially affected by protein kinase A, consistent with separate, yet convergent, signaling pathways. Immunoprecipitation experiments revealed that treatment with okadaic

acid increased the phosphorylation of the antiport, and the use of inhibitors indicated that this effect was not due to protein kinases A, C, or CaM. Although phosphorylation of ancillary proteins may also be involved, direct phosphorylation of the antiport seems a likely mechanism to account for the alkaline shift in its pH dependence.

H⁺ Channels

Passive conductive mechanisms for H^+ or its equivalents (OH^-, HCO^{3-}) have not been considered important regulators of intracellular pH, because the electrochemical gradient would normally drive acid equivalents into the cells. Phagocytes are unique, however, in that activation by invading microorganisms not only tends to lower cytosolic pH, but also depolarizes the plasma membrane, thus reversing the proton motive force. Experiments were therefore undertaken to determine whether an H^+ conductance is activated in neutrophils when exposed to agents that mimic microbial infection. Using fluorimetric determinations of intracellular pH, Dr. Grinstein and his colleagues found the conductance in unstimulated cells to be low and unaffected by moderate changes in membrane potential. In contrast, a sizable H^+ conductance was unmasked when the cells were stimulated with protein kinase C agonists or with chemoattractant peptides.

The existence of an H^+-selective conductance was verified electrophysiologically, using the whole-cell configuration of the patch clamp. In human neutrophils and in HL-60 cells differentiated along the granulocytic lineage, an outward H^+ current was detected. This current was voltage-gated and was exquisitely sensitive to the intracellular pH. Preliminary results indicate that the permeability pathway mediating the H^+ current is extremely selective and displays a sharply rectifying behavior, allowing the passage of acid equivalents out of, but not into, the cells. The equivalence of the fluxes measured elec-

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trically and by fluorescence, the molecular details of their activation, and their role in the regulation of internal pH are being actively investigated.

Vacuolar-type H⁺ Pumps

Studies in Dr. Grinstein's laboratory also addressed the role of H⁺ pumps in the regulation of the pH of the cytosol and other intracellular organelles. Like antiports and channels, vacuolar H⁺ pumps were found to be active in cytosolic pH regulation in phagocytes. Recovery from an acid load was found to be accompanied by extrusion of acid from the cells, consistent with a plasmalemmal location of the pumps. In neutrophils, pumping was only evident following stimulation with phorbol esters or chemoattractants, suggesting migration of intracellular pumps to the membrane or functional unmasking of resident plasmalemmal pumps.

Vacuolar-type H⁺ pumps were also found to be central to the generation and maintenance of an acidic interior in phagosomes. A low phagosomal pH is essential for effective microbial killing. The determinants of the intraphagosomal pH were studied *in situ*, recording the emission of covalently fluoresceinated bacteria ingested by macrophages. The phagosomal membrane was found to be comparatively tight, although not entirely impermeant to H⁺. A counterion conductance, which supports the rheogenic movement of H⁺ via leaks or pumps, was also detected. Both anions and cations were found to contribute to the counterion pathway. The magnitude of the counterion flux suffices and, in fact, greatly exceeds the rate of H⁺ pumping in the steady state.

These findings imply that the phagosomal membrane potential is negligible and therefore not a major contributor to the establishment of intraphagosomal pH. The rate of active H⁺ pumping was found to decrease steeply as the phagosomal lumen became acidified. Therefore it appears that the intrinsic pH sensitivity of the vacuolar H⁺ pumps, which likely reflects a kinetic or allosteric effect, is the primary determinant of the intraphagosomal pH. It is thus conceivable that the differential pH of distinct endomembrane compartments is dictated by the H⁺ pumps themselves, as a result of the existence of varying pump isoforms or the presence of allosteric inhibitors or activators. Experiments are in progress to resolve these alternatives.

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STRUCTURE/FUNCTION ANALYSIS OF DRUG EFFLUX PUMPS ENCODED BY THE MULTIDRUG-RESISTANCE GENE FAMILY

Philippe Gros, Ph.D., *International Research Scholar*

Multidrug resistance (MDR), the phenomenon by which tumor cells develop resistance to a wide range of structurally unrelated chemotherapeutic drugs, is caused by the overexpression of P-glycoprotein (P-gp). P-gp binds photoactivatable analogues of ATP and cytotoxic drugs and is thought to function as a membrane-associated ATP-driven drug efflux pump. P-gp is encoded by a small family of closely related genes, termed *mdr* or *pgp*, that share considerable sequence homology and common ancestral origins. There are three *mdr* genes in rodents (*mdr1, mdr2, mdr3*) and two in humans (*MDR1, MDR2*).

Full-length cDNA clones corresponding to the three mouse genes have been cloned and characterized. The three P-gps are highly homologous (80–85% sequence identity), with identical predicted structural features that include 12 transmembrane (TM) domains and two nucleotide binding sites. Each P-gp is formed by two homologous halves that show sequence conservation with a large group of bacterial transport proteins participating in the import and export of specific substrates in *Escherichia coli*.

The *mdr* gene family is itself part of a larger gene family that includes the *STE6* gene of the yeast *Saccharomyces cerevisiae*, the gene responsible for the transmembrane transport of the "**a**" mating pheromone; the *pfmdr1* gene of the malarial parasite *Plasmodium falciparum*, associated with chloroquine (CLQ) efflux in this parasite; and in humans, the *CFTR* chloride channel gene, in which mutations cause cystic fibrosis, and the *TAP-1/TAP-2* family, encoding peptide pumps participating in antigen presentation by T lymphocytes. Therefore the *mdr* supergene family codes for transport proteins that act by the same mechanism on different types of substrates. Transfection and overexpression of full-length *mdr* cDNAs into otherwise drug-sensitive cells show that *mdr1* and *mdr3* can directly convey MDR, while *mdr2* cannot.

The mechanism by which P-gp mediates drug efflux remains unknown. So do the discrete protein domains and amino acid residues implicated in recognition of structurally heterogeneous MDR drugs. Answering these questions is a necessary prerequisite to the rational design of new cytotoxic drugs or experimental strategies aimed at blocking or by-passing the action of this pump. In the absence of a three-dimensional structure, the structure/function analysis of P-gp has focused on the creation and characterization of chimeric and mutant P-gps.

Identification of Protein Domains Implicated in Drug Recognition

P-gps encoded by mouse *mdr1* and *mdr3* confer distinct drug-resistance profiles to transfected cells. Both clones confer comparable levels of resistance to vinblastine (VBL), while *mdr3* confers preferential resistance to actinomycin D (ACT), and *mdr1* to colchicine (COL). To identify protein domains implicated in the preferential drug resistance encoded by either parental *mdr* clone, homologous protein domains were exchanged in a series of 16 hybrid cDNA clones. While all chimeric clones conferred similar levels of VBL resistance, the levels of ACT and COL resistance conferred by the various chimeras were heterogeneous, being either similar to the parental *mdr1* or *mdr3* clones or, in many cases, intermediate between the two. Only those chimeric proteins carrying segments overlapping both the amino and carboxyl sets of TM domains of the respective parent conveyed the full preferential drug-resistance profile of this parent.

These results suggest that 1) both parental proteins transport VBL by the same mechanism, which involves protein determinants that are conserved in both parents and that can be interchanged in chimeric proteins, and 2) the preferential drug-resistance profiles encoded by parental *mdr1* or *mdr3* involve several determinants associated with TM domains from both homologous halves of P-gp.

A simple Ser → Phe substitution at position 941 (*mdr1*) or 939 (*mdr3*), within predicted TM11, was shown to have a dramatic effect on the overall activity and substrate specificity of the two pumps. The modulating effect of this mutation was very strong for COL and adriamycin (ADR) but only moderate for VBL. For *mdr1*, the Ser → Phe replacement produced a unique mutant protein that retained the capacity to confer VBL resistance but lost the ability to confer ADR or COL resistance.

These results suggest that ADR/COL and VBL may have distinct binding sites on P-gp and that the serine residue within TM11 plays a key role in the recognition and transport of the former drugs by P-gp. The same residue has also been found mutated in the *pfmdr1* gene from CLQ-resistant isolates of the human malarial parasite *Plasmodium falciparum*. Together these studies indicate that the TM11 domain

of *mdr* and *mdr*-like genes is critical for drug recognition and transport.

Construction of Model Substrates for P-gp

The high degree of hydrophobicity and the presence of a positive charge at neutral pH are two major common characteristics of MDR drugs. In an effort to identify the chemical determinants of MDR drugs that are essential for recognition by P-gp, simple lipophilic cations such as tetraphenylphosphonium (TPP$^+$) and tetraphenylarsonium (TPA$^+$) were tested and shown to be transported by P-gp. The progressive replacement of phenyl groups or introduction of long aliphatic side chains was shown to reduce their affinity for P-gp. It appears from these preliminary studies that the ability of the phenyl groups to delocalize the charge in the molecule is an important determinant for both cytotoxicity and P-gp recognition. By opposition to known MDR drugs, the relatively simple structure of these compounds renders them very easily amenable to extensive chemical modification, and Dr. Gros and his colleagues have started to analyze additional derivatives of TPP$^+$.

Complementation of the Yeast *mdr* Homologue *STE6* by Its Mammalian Counterpart

The yeast *Saccharomyces cerevisiae* homologue of *mdr*, *STE6*, is the membrane transporter mediating export of the a-factor mating peptide. Yeast *MATa* cells carrying an *ste6* deletion produce no extracellular a-factor and therefore are defective in mating. Expression of a full-length cDNA clone for mouse *mdr3* in a yeast *ste6* deletion strain was shown to restore the ability to export a-factor and to mate. A mutation (a Ser → Phe substitution at position 939 in TM11) known to affect the activity of the *mdr3* gene product abolished its ability to complement the yeast *ste6* deletion. These experiments show that P-gp can transport peptides in yeast cells and perhaps also in normal mouse tissues. In addition, the functional expression of P-gp in yeast cells should 1) greatly facilitate the structure/function analysis of P-gp by site-directed mutagenesis, including the analysis of possible intramolecular interaction in P-gp by selection of second-site mutations; 2) permit production of large amounts of P-gp

for functional and structural studies; and 3) enable the design and testing of a-factor peptide analogues as potential blockers of P-gp function.

Other Activities

The mouse mutation *splotch* (*Sp*) blocks closure of the neural tube during neurogenesis, resulting in spina bifida and exencephaly, two common birth defects in humans. It has recently been shown that the *Sp* mutation (*Sp^{2H}*) is caused by a deletion in the paired box of the *Pax-3* gene, a member of a new family of transcription factors (*Pax*) expressed exclusively during embryogenesis. This deletion also removes the entire homeodomain and transactivating domain of *Pax-3*. These results identify a key role for *Pax-3* in the normal and perhaps abnormal development of the nervous system in mammals.

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TRANSLATIONAL REGULATION BY BACTERIOPHAGE λ

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There are few documented cases of control of host-cell protein synthesis by bacteriophage. Dr. Guarneros and his colleagues have focused their research on translational control events in which λ phage–directed transcripts act on protein synthesis at a step controlled by peptidyl-tRNA hydrolase (Pth).

Bacteriophage λ is unable to grow vegetatively on bacterial mutants for the *pth* gene. Phage mutants that compensate the hydrolase defect defined several sites in the λ genome. In an effort to understand this relationship, the phage *bar* mutation and the bacterial *pth* mutations have been studied.

It has been known for some time that Pth is essential for the cell. Bacteria carrying a thermosensitive mutation in *pth* accumulate peptidyl-tRNAs, stop protein synthesis, and die at the nonpermissive temperature. It is accepted that Pth scavenges peptidyl-tRNAs that fall off the ribosomes in translational editing. This view assumes that the accumulation of peptidyl-tRNAs is toxic for the cell.

Characterization of the Bacterial and λ Products Involved in the Regulatory Interaction

Sequencing analysis in the laboratory of Dr. Guarneros identified a translational open reading frame with the *pth* gene. Mutations that conferred the phage exclusion phenotype were 1-bp substitutions in *pth*. The Pth activity was isolated and purified. The properties of the polypeptide corresponded to the predicted global amino acid composition, amino-terminal sequence, molecular weight, and isoelectric pH. In addition, in collaboration with Dr. Richard Buckingham in Paris, it was shown that the assigned polypeptide had Pth activity.

The λ mutations that overcome the Pth defect were located to several *bar* loci. The sequence analysis of mutations in two discrete loci defined a nearly identical 16-bp DNA segment containing dyad symmetry. Genetic evidence suggested that the inhibition of λ growth in mutant *pth* cells required transcription of the *bar* region. This supposition proved to be correct in a plasmid system.

λ Bar Transcription May Be Lethal to *pth* Bacteria

Plasmid constructs containing a λ *bar* sequence under an active promoter phenotypically parallel phage exclusion in Pth-defective cells. Transcription through wild-type λ *bar* sites was lethal to Pth-

defective (but not to wild-type) cells, whereas transcription of the mutant *bar* sites was harmless. This result does not necessarily imply that phage growth inhibition occurs by increased *pth* mutant lethality.

Plasmid clones carrying *bar* sequences as small as 21 bp and containing the 16-bp core caused lethality of *pth* mutants. Most likely, transcripts are the active molecules, since there is no evidence of translatability of these sequences. Transcription of *bar* is followed by a general arrest of protein synthesis in the cell. Since RNA synthesis is not blocked, the inhibition must be a post-transcriptional effect. It is likely that inhibition of protein synthesis causes the lethality of Pth-defective cells.

A Model for λ Regulation of Protein Synthesis

The Bar transcripts may stop protein synthesis by interfering with translation termination. Drs. Emanuel Murgola, Albert Dahlberg, and their collaborators have implicated antiparallel pairing of 16S ribosomal RNA and UGA termination codons in mRNA as part of the normal process of peptide synthesis termination. Such base-pairing facilitates peptidyl-tRNA hydrolysis by preventing amino acid misincorporation at UGA (suppression).

Drs. Murgola and Guarneros have proposed that Bar RNA competes with the UGA codons in mRNA for association with ribosomal 16S RNA. This would leave UGA codons in mRNA available for suppression, therefore blocking efficient peptidyl-tRNA hydrolysis. The proposal includes the participation of Pth in the translation termination step as a result of the association of the hydrolase with Bar regulation. Inspection shows that the transcripts of the core *bar* sequences contain a UGA codon within a five-nucleotide tract capable of antiparallel pairing with ribosomal 16S RNA. Mutant Pth may cause a defect in polypeptide termination by facilitating Bar RNA–16S RNA interaction. Results of non-sense suppression in Pth-defective bacteria and of UGA-specific Bar-mediated suppression in wild-type cells support the basic predictions of the model.

Role of Bar Regulation in λ Biology

The results discussed above for the *bar* plasmids in Pth-defective bacteria cannot be directly applied to explain the role of Bar RNA in λ biology. However, assuming either of the two functions advanced for Pth, scavenging of free peptidyl-tRNAs or translation termination, Dr. Guarneros and his colleagues

have proposed two possible roles for Bar regulation: 1) Bar RNA controls the relative levels of specific tRNAs to fit the frequency of codon usage in λ, which assumes that Bar RNA regulates Pth activity differentially for distinct peptidyl-tRNAs; and 2) Bar RNA acts on termination of polypeptide chains directed by phage transcripts.

Among λ genes, UGA is the more frequent termination codon (and not UAA as in the host cell). In addition, it is frequently the case in the λ genome that the UGA termination codon of a gene partially overlaps with the AUG initiation codon of the next gene in the tetramer AUGA. Since the Bar core transcript contains AUGA and allegedly interacts with ribosomal 16S RNA, this suggests a role in polypeptide chain termination/initiation events for the overlapping genes.

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MOLECULAR GENETICS OF PHOTOSYNTHESIS AND CARBON ASSIMILATION IN PLANTS

Luis R. Herrera-Estrella, Ph.D., *International Research Scholar*

Photosynthesis and Carbon Assimilation

Photosynthesis and carbon assimilation are the most important biochemical and molecular events in the life cycle of higher plants and, indeed, the key to the provision of nutrients for the whole food chain. Solar energy is first collected in the chloroplasts of photosynthetic tissues, mainly in leaves, by light-harvesting antennas composed of chlorophyll and protein molecules (chlorophyll *a/b*–binding proteins). The energy obtained is then used to convert atmospheric CO_2 into triose phosphate molecules. These three-carbon molecules proceed through a series of reactions, called the Calvin-Benson cycle, that culminates in the production of sugars, from which all the organic molecules that are required for the life of plants are synthesized.

Triose phosphate molecules are converted in the cytoplasm of photosynthetic or source cells into sucrose, which is translocated through the phloem to feed all nonphotosynthetic or consumer tissues— roots, flowers, seeds, tubers, etc. Assimilated carbon is temporally or permanently stored in the form of starch in both source and consumer tissues. The starch stored in seeds or tubers serves as the major source of carbon and energy for the germination and development of new plants.

The light-dependent production of ATP and NADPH, the reductive assimilation of CO_2, and sucrose and starch synthesis are interlinked and interdependent. *In vivo*, these processes must be coordinated at both the biochemical and genetic level (i.e., at the level of gene expression). The balance between the efficiency of CO_2 fixation, sucrose translocation and uptake, and assimilation of sucrose in consumer tissues plays a fundamental role in determining the productivity of any given plant species. This balance is affected by both genetic determinants of the individual and its interaction with the environment.

Characterization of DNA Sequences Involved in the Light Regulation and Tissue-Specific Expression of *cab* Genes

For the past several years, Dr. Herrera-Estrella's laboratory, in collaboration with Dr. June Simpson, has identified cis-acting sequences involved in the regulation of *cab* and rubisco genes. These sequences have been shown to be responsible for the light-inducible and tissue-specific expression of both types of photosynthetic genes. Deletion analysis using transgenic plants has allowed the identification of a 247-bp DNA sequence that can regulate a heterologous promoter in a light-inducible and tissue-specific fashion and contains positive and negative regulatory elements.

During the past year the effort of this group has focused on identifying the trans-acting factors that interact with this 247-bp sequence and the DNA motif to which these trans-acting factors bind. Two protein factors were identified that bind to these

regulatory regions. One of the factors, ABF-1, was found to be present in photosynthetically active tissues but not in etiolated or root tissue.

The second factor, ABF-2, is present in all tissues analyzed and binds to a DNA sequence that contains a direct heptamer repeat, TCTCAAA. It was found that both repeats are essential for binding. ABF-2 is evolutionarily conserved in several plant species. Computer analysis showed that the TCTCAAA motif is present in the 5'-flanking region of several highly inducible plant genes and that the distance between the two motifs required for binding seems to be important for their function. The next important step is to determine which combination of these DNA motifs determines the light-inducible properties of photosynthetic genes. In addition, protocols for the transformation of tomatillo (*Physalis ixocarpa*) are being developed to enable the study of the regulation of photosynthetic genes during fruit development.

Molecular Approaches to Understanding the Function of Chaperonin 60 Genes

Chaperonin 60 α and β polypeptides have been implicated in the assembly of rubisco, acting as molecular chaperones. However, apart from the strong indirect evidence supporting this role for chaperonin 60 (cpn60) polypeptides, no direct evidence is available to confirm the hypothesis. To study the function of these polypeptides, Dr. Herrera-Estrella's group has isolated the *Arabidopsis thaliana* genes encoding the cpn60 β polypeptides.

In this plant the cpn60 β gene family is composed of three functional genes. In the past year, Dr. Herrera-Estrella's laboratory has focused on determining the tissue-specific expression directed by the 5'-flanking region of the cpn60 β genes and on studying their function by generating transgenic plants harboring cpn60 β antisense gene constructs. Using chimeric genes in which the coding sequence of the bacterial β-glucuronidase gene is under the control of cpn60 β promoter, the group found that these genes have high levels of expression in the mesophyll cells of photosynthetic tissues and in the male and female reproductive organs. A developmentally regulated expression was also observed in stems, where expression is initially detected in the peripheral-photosynthetic area but is later observed only in vascular tissue. This is an important step in the understanding of the function of cpn60 β genes, because their expression in nonphotosynthetic tissues suggests that the encoded polypeptide must have functions other than the assembly of rubisco.

Transgenic plants expressing antisense cpn60 β RNA show different degrees of stunting and have chlorotic mature leaves. In contrast to what could be expected from the proposed function of cpn60, the antisense transgenic plants have higher levels of rubisco activity than nontransformed control plants. The most conspicuous biochemical alteration in these plants is that the level of sucrose phosphate synthase is drastically reduced. Starch content analysis and electron microscopy showed that the mature leaves of these plants contain large quantities of starch accumulated in the plastids of the cells. This finding suggests that cpn60 β polypeptides may not be involved in rubisco assembly but that they affect either the systems involved in the transport of triose phosphate molecules from the chloroplast to the cytoplasm or in the cytoplasmic conversion of triose phosphate molecules into sucrose.

Molecular Analysis of Sucrose Phosphate Synthase Genes

Sucrose phosphate synthase (SPS) could be a key enzyme in the assimilation of photosynthates. Dr. Herrera-Estrella and his colleagues, in collaboration with the group of Dr. Horacio Pontis in Argentina, have sought to isolate and characterize the genes encoding SPS. Using antibodies and polymerase chain reaction technology, cDNAs encoding SPS have been isolated. SPS is present as a single-copy gene in maize, rice, and *Arabidopsis*. Molecular characterization of these genes and studies aimed to investigate the importance of this enzyme in carbon assimilation, using transgenic plants containing sense and antisense gene constructs, are in progress.

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MOLECULAR AND MUTATIONAL ANALYSIS OF MAMMALIAN GENES CONTROLLING PATTERN FORMATION

Alexandra L. Joyner, Ph.D., *International Research Scholar*

One strategy that has been very effective in identifying mammalian genes required for pattern formation has been to use cross-species sequence conservation to clone related genes from vertebrates and invertebrates. A number of families of mammalian genes have been identified in this manner, based on their homology to *Drosophila* genes that control the process of segmentation. These families include the *Wnt* genes that are homologues of the *Drosophila wingless* (*wg*) gene, the *En* genes that are similar in structure to the *Drosophila engrailed* (*en*) gene, the *Pax* genes that contain a conserved paired box domain that is found in the *Drosophila paired* (*prd*) and *gooseberry* (*gsb*) genes, and the *Gli* genes that have homology to the *Drosophila cubitus interruptus* (*Ci*) gene. Analysis of gene expression patterns and limited mutant analysis have indicated that these mammalian genes play important roles in pattern formation during gastrulation and organogenesis. In *Drosophila*, the *wg*, *en*, *prd*, *gsb*, and *Ci* genes have been shown to interact during segmentation in common genetic pathways.

The expression patterns of the mouse homologues of these genes have shown that they may also interact in a similar manner in mammals. Thus it is possible that not only the genes but also the genetic pathways have been conserved through evolution. Dr. Joyner and her colleagues have concentrated on determining the roles of the *En* genes in development and, more recently, on examining whether they interact with the *Wnt*, *Pax*, and *Gli* genes.

Function of Mouse *En* Genes

Mice, humans, and chickens all contain two *engrailed* genes, *En-1* and *En-2*, that are expressed in a very similar band of cells across the developing midbrain-hindbrain border, beginning shortly after formation of the neural ectoderm. In the adult the *En* genes are expressed in specific neurons in the cerebellum and pons region, rather than in a spatially restricted domain. An *En-2* mouse mutant was made by gene targeting in embryonic stem (ES) cells, and mice homozygous for this mutation, *En-2*hd, show an abnormality in patterning of the folds of the cerebellum, although they are viable, fertile, and show no obvious behavioral defects. Recently ES cells containing a mutation in *En-1* have been made and chimeras have been produced from the cells. If mice heterozygous for this mutation are obtained from the chimeras, they will be bred to test

whether *En-1*, unlike *En-2*, is essential for development and/or whether there is a level of redundancy of function between *En-1* and *-2*.

In order to characterize further the *En-2*hd mutant phenotype, the developmental profile of presentation of the defect was characterized. By analyzing the cerebellums of mutant and wild-type mice during the first two weeks after birth, the time at which the cerebellar folds are produced, the adult phenotype was shown to arise from abnormalities that occur during the first week of postnatal development. The defects include fusion of folds, abnormal placement of folds, and transformations of one fold to another. These types of defects are reminiscent of those that occur in *Drosophila en* mutants. In an attempt to understand the mechanism underlying this patterning defect, Dr. Joyner and her colleagues are analyzing the expression of *En-1* and *En-2* in the postnatal cerebellum.

The *En* Genetic Pathways

To identify the upstream gene(s) that regulates the spatially restricted expression of the *En* genes, the laboratory is carrying out a promoter analysis in transgenic mice. By making reporter constructs (containing DNA fragments 5' and 3' to *En-2*, a minimal promoter, and the *lacZ* gene) and analyzing β-galactosidase expression in transgenic embryos, a 1-kb fragment that contains a mid-hindbrain enhancer has been identified. A similar DNA sequence has been identified upstream of the human *En-2* gene based on DNA sequence similarities. A biochemical analysis of the embryonic brain proteins that bind the mouse *En-2* enhancer fragment will be undertaken, with the aim of cloning the genes responsible for regulating *En-2*, and possibly *En-1*, expression in the embryo.

To determine whether the *Wnt*, *Gli*, and *Pax* genes interact with the *En* genes, Dr. Joyner and her colleagues are analyzing comparative expression in wild-type and mutant embryos and characterizing double mutants. The initial studies have concentrated on the developing cerebellum. To facilitate this analysis, the mouse *Gli*, *Gli-2*, and *Gli-3* genes were cloned and their expression patterns determined in the embryo, in collaboration with Dr. Robert Holmgren (Northwestern University). In addition, an existing mouse developmental mutant, *Extra toes*, was shown to contain a deletion of *Gli-3*. The phenotype of this mutant and double mutants

with *En-2ʰᵈ* will be analyzed, as well as expression of the three *Gli* genes in the postnatal cerebellum.

Two approaches have been taken, in collaboration with Dr. Andrew McMahon (Roche Institute), to analyze possible interactions of the *En* and *Wnt* genes. One approach is to analyze the expression of the *En* genes in embryos mutant for *Wnt-1*. These studies have shown that, as in the fly, the *En* genes do not require *Wnt-1* to be activated, but may require *Wnt-1* for maintenance of *En* expression. The second approach has been to make double mutants and analyze the cerebellar phenotype.

The Mouse *Mash* Genes

A number of genes critical for development of the nervous system in *Drosophila* have been identified. The *achaete-scute* complex (AS-C) family of genes is required both for determination of neuroblasts from ectoderm and for specific differentiation of certain sensory neurons. These genes are all thought to be transcription factors that dimerize and bind DNA through a basic helix-loop-helix (bHLH) protein motif. To determine whether these genes play a similar role in development of the mammalian nervous system, Dr. Joyner and her colleagues cloned two mouse genes, *Mash-1* and *-2* (*Mash*: mammalian *achaete-scute* homologue), that contain an AS-C type of bHLH domain, based on their homology to the *Drosophila* AS-C genes, and Dr. David Anderson and his colleagues (HHMI, California Institute of Technology) cloned two *Mash* genes of the rat. *Mash-1* is expressed primarily in proliferating cells of the embryonic nervous system, whereas *Mash-2* expression is restricted to proliferating cells of the trophectoderm extraembryonic lineage. Mice heterozygous for a targeted deletion of *Mash-1* have been made and will be bred to determine whether

Mash-1 is essential for development of the nervous system.

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PRACTICAL APPLICATIONS OF REPLICATABLE RNAs AND RNA ENZYMES IN THE DIAGNOSIS OF INFECTIOUS DISEASES

Paul M. Lizardi, Ph.D., *International Research Scholar*

Epidemiological monitoring of infectious diseases is a high priority in developing countries. The list of diseases producing significant mortality and morbidity includes AIDS, malaria, amebiasis, tuberculosis, hepatitis, trypanosomiasis, typhoid fever, diverse intestinal infections, and recently cholera. There is a need for techniques that will permit the

rapid and reliable detection of infectious agents, so that epidemiological monitoring and patient management can be more effective. The use of the polymerase chain reaction (PCR) for the detection of pathogens has demonstrated the power of nucleic acid amplification as a diagnostic tool. Hence there is considerable interest in exploring other nucleic

acid amplification schemes that may have potential for the design of rapid, automatable diagnostic assays.

The work of Dr. Lizardi is concerned with exploiting RNA amplification as a tool for the development of rapid and sensitive diagnostic assays for pathogens. Dr. Lizardi's work in Mexico is being carried out in collaboration with Dr. Fred Kramer (Public Health Research Institute, New York City) and Dr. Jack Szostak (Massachusetts General Hospital, Boston).

Amplifiable RNA Probes

A specific class of RNA molecules known as replicatable RNAs can be amplified exponentially at a constant temperature of 37°C, generating millions of copies of each molecule. The best-characterized RNA amplification reaction, catalyzed by the enzyme Q-beta replicase, is unique because parent and daughter RNA single strands are forced apart as they are synthesized, in contrast to DNA-dependent polymerization reactions, in which the two strands remain annealed.

Assays have been designed in which replicatable RNAs are used as reporters for the presence of nucleic acid targets. In such assays a replicatable RNA containing a probe insert is incubated with a biological sample under suitable hybridization conditions that allow specific binding of the probe domain to its intended target. A subsequent washing step removes those probes that did not bind to the target. Finally, the bound probes are released from their target and incubated in the presence of Q-beta replicase. The ensuing replication reaction generates as many as 100 million copies of each RNA probe in ~20 min, and the resulting RNA mass is readily quantitated by fluorescence staining. The intensity of the fluorescent signal is proportional to the number of targets present in the original sample.

The scheme outlined above, known as RNA probe amplification, has been used to detect HIV-1 (human immunodeficiency virus type 1) RNA targets in human blood, and has been demonstrated to have a limit of detection of ~10,000 molecules of viral nucleic acid. This limit is artificially imposed by the presence of replicatable RNA probes that bind nonspecifically to surfaces in the assay medium.

RNA Binary Probes That Can Be Amplified After Ligation

It should be possible to improve significantly the limit of detection in Q-beta–amplified assays by using probes that cannot be amplified by the replicase until an additional enzymatic step involving target

recognition has occurred. This requirement can be met by using a binary probe scheme, in which a ligase-mediated joining reaction provides additional specificity in target recognition.

Using HIV-1 integrase mRNA as a model target, it has recently been possible to implement a binary probe system that generates an amplifiable signal only in the presence of the viral mRNA target. The binary probe system consists of two RNA molecules that contain probe segments complementary to the target sequence domain, as well as additional sequences derived from a replicatable RNA. Neither of the two binary probe molecules is replicatable; however, they can become replicatable if joined covalently to form a single molecule. The RNA probe segments are designed to bind to the integrase mRNA target so that their 3' and 5' ends are perfectly juxtaposed. After binding, the ends can be joined in a reaction catalyzed by a novel RNA enzyme known as a ribozyme ligase, which was constructed in Dr. Szostak's laboratory by modification of the type I intron of *Tetrahymena*. This enzyme catalyzes covalent joining of RNA termini that are perfectly aligned by base-pairing on a complementary polynucleotide.

A number of technical problems remain to be solved to achieve optimal sensitivity and specificity in the binary probe assay. The ligation step involving the ribozyme ligase is still relatively inefficient, since only 25% of the ligation-competent binary probe molecules are joined in a 1-h incubation. An actual assay would require a ligation efficiency of ~75%. Recent progress in the ability to generate novel RNA enzymes suggests a possible solution to this problem.

Directed Evolution of More-Efficient Ribozymes by *in Vitro* Selection

It is now possible to generate novel ribozymes with altered catalytic efficiency by directed evolution *in vitro*. Chemical synthesis is used to generate a mutant pool of 10^{12} molecules of DNA, each containing a sequence variant of the ribozyme. T7 RNA polymerase is then used to generate RNA copies of the DNA, thus creating more than 10^{12} different ribozyme species. The ribozymes are incubated for a short time in a test tube under RNA ligation conditions. Those ribozymes that succeed in carrying out the ligation reaction are amplified by subsequent cycles of reverse transcription followed by PCR, and the entire process is repeated several times. After four cycles of selection, a relatively small number of mutant ribozyme sequences are present in the DNA population, instead of the original 10^{12}. The mole-

cules are then sequenced and compared with the original parental ribozyme.

Experiments in progress are directed toward the generation of improved ribozyme ligases specifically suited for binary probe assays. A threefold improvement in ligation efficiency seems an attainable goal and may permit the implementation of RNA probe amplification assays capable of detecting as few as 200 molecules of target.

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EARLY DEVELOPMENT IN NEMATODES

James D. McGhee, Ph.D., *International Research Scholar*

Dr. McGhee's laboratory focuses on the question of how specific genes become expressed only in certain lineages of the developing embryo. The model organism under study is the nematode *Caenorhabditis elegans*, a small free-living worm that has numerous experimental advantages, such as a defined cell lineage, small number of cells, and accessibility to both micromanipulation and manipulation by classical genetics. The laboratory has recently begun to study other nematodes as well—for example, the reasonably closely related nematode *Caenorhabditis briggsae* and *Ascaris suum*, the evolutionarily distant intestinal parasite of pigs. Nematodes are a fascinating group of organisms in which to study patterns of gene expression, since their basic body plan and developmental strategies have remained largely unchanged over hundreds of millions of years of evolution, even in the face of large changes in DNA sequence.

Most of the work in the laboratory involves gut development, since the nematode intestine is a particularly simple lineage. It is established when the embryo has only eight cells, and it appears to develop in the absence of major interactions with other cells. As an experimentally convenient molecular marker of gut differentiation, the animal uses an intestine-specific digestive enzyme, the product of a carboxylesterase gene called *ges-1*, which is homologous to esterases present in insects and mammals. The experimental approach most used at present is to modify the cloned *ges-1* DNA *in vitro*, inject it into mutant worms that do not express esterase activity, and then stain these transformed worms to see in which cells the *ges-1* activity appears. Past work has shown that *ges-1* control is unexpectedly complex. In particular, the gene appears capable of being expressed in certain nongut lineages. This nongut expression is revealed when specific regions of *ges-1* have been deleted. The working model in the laboratory is that lineage-specific gene expression in-volves not only activators in the expressing lineage (in this case, the gut) but also repressors in nonexpressing tissues (in this case, primarily muscle cells of the pharynx).

Efforts are now focused on identifying the sites of action of these regulatory molecules, both putative repressors and putative activators. One approach that has been used is to investigate the gene sequences and gene control mechanisms in other nematodes. To this end, the *ges-1* homologue has been cloned from the related nematode *C. briggsae*. Coding sequences have been highly conserved; but apart from the coding regions, except for one short sequence element, little obvious similarity can be detected. Yet, despite these sequence differences, and although the two worms diverged an estimated 40 million years ago (and possibly even earlier), the *C. elegans* gene appears to be expressed correctly when transformed into *C. briggsae* and, to a good approximation, the *C. briggsae* gene appears to be expressed correctly when transformed into *C. elegans*. Detailed sequence examinations, deletions, and substitutions are now being used to define functional sites unambiguously.

The laboratory has also established an experimental system in which to investigate the biochemistry of early *C. elegans* development. Parent worms are grown in the presence of the deoxynucleotide analogue fluorodeoxyuridine, and development of the next generation of embryos is completely blocked at a point in development just after *ges-1* expression has been initiated. Protein binding factors have been detected in nuclear extracts prepared from this homogeneous population of embryos. Two factors have been identified that interact with a region of the *ges-1* gene believed to be the "gut activator," and a different factor has been identified that binds to the one sequence highly conserved between *C. elegans* and *C. briggsae*.

Reasonably large quantities of unfertilized oo-

cytes have also been produced, using a temperature-sensitive sperm-defective mutation. The laboratory has shown that the factors binding to the gut activator are not present in oocytes; however, the factor binding to the conserved site is present in oocyte cytoplasm and presumably reflects a maternal contribution to *ges-1* control. The present aims are to use this biochemical system to clone the genes for the interacting factors, as well as to investigate the behavior of the factors in extracts made from mutant worms in which development has been perturbed.

Dr. McGhee and his colleagues are also investigating early development in the much larger and more slowly developing parasitic nematode *Ascaris suum*. As a first step in characterizing the system, they have measured the rates of DNA synthesis in early cleavage cycles. The *Ascaris* cell cycles contain a G_2 phase as predominant, unlike the cell cycles of the rapidly developing *C. elegans* embryos, previously shown to consist only of alternations between DNA synthesis and mitosis. The laboratory has purified an esterase enzyme from *Ascaris* intestines and has obtained sufficient amino acid sequence information to identify it as indeed the *ges-1* homologue. However, unlike the gut-specific *ges-1* expression patterns in *Caenorhabditis*, the *Ascaris* *ges-1* gene appears to be expressed in most if not all tissues of the body. The hope is that such comparative studies of gene expression patterns will provide insight into gene control mechanisms and their evolution.

Another direction the laboratory has taken is to clone and characterize genes in *C. elegans* that, judging from work in other organisms, are certain to be important regulatory molecules. For example, a *C. elegans* homeobox gene, *ceh-10*, has been cloned and sequenced. This gene bears striking similarity to genes expressed in the central nervous system of mammals. The group has also cloned the *C. elegans* homologue of the *Drosophila* gene *forkhead* and of the mammalian liver-specific transcription factor HNF-3. Both these latter genes are specific to endoderm, although development differs widely in insects and mammals. Thus it will be of special interest to see where the *C. elegans* homologue of *forkhead* and HNF-3 is expressed during nematode development. The aim of these experiments is to see how highly conserved transcription factors are distributed among embryonic germ layers in widely different animals. It is hoped that this will reveal general and deep-seated mechanisms of embryonic development and will even shed light on the evolutionary origin of multicellular animals.

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Article

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CYTOKINE REGULATION OF IMMUNE RESPONSES

Tim Mosmann, Ph.D., *International Research Scholar*

The immune system is capable of responding to an infectious agent by invoking many different effector functions. Only certain mechanisms will be effective against a particular pathogen, and so it is not surprising that the choice of mechanism is carefully regulated by the immune system. Much of this regulation is mediated by cytokines secreted by cells of the immune system, particularly T cells. Dr. Mosmann found several years ago that different subsets of T cells secrete different sets of cytokines when stimulated with antigen, and several investigators showed that these cytokine patterns are responsible for the unique functions of each subset.

Two major types of T helper cell, TH1 and TH2, are mainly responsible for inducing cell-mediated and antibody responses, respectively. The TH2-enhanced production of antibodies is particularly useful in combating infections with extracellular agents—for example, by neutralizing bacterial and viral attachment sites or blocking the action of toxins. In contrast, the cell-mediated delayed-type hypersensitivity (DTH) response results in the local recruitment and activation of effector cells, such as macrophages and granulocytes, that can efficiently kill cells infected with intracellular pathogens.

The unique cytokines of TH1 or TH2 cells are major regulators of the functions of these cells. For example, TH2 cells evoke allergic responses by the secretion of interleukin-4 (IL-4), which induces immunoglobulin E production, and IL-5, which in-

creases the number of eosinophils. In contrast, TH1 cells secrete interferon-γ and lymphotoxin, which activate the killing functions of macrophages and granulocytes.

A few years ago a cDNA library made from induced TH2 cells was used to isolate a clone, P600, that was expressed in activated but not resting TH2 cells. The P600 sequence had no homology to other sequences in nucleic acid databases, and the open reading frame potentially encoded a 131–amino acid protein that contained a short hydrophobic sequence at the amino terminal, suggesting a secreted protein. P600 mRNA was synthesized after activation of TH2 but not TH1 cells. All of this information is consistent with the hypothesis that the P600 protein is a new TH2-specific cytokine. Because all of the other TH2-specific cytokines play a major role in the unique functions of TH2 cells, Dr. Mosmann's laboratory has been investigating the functions of P600 to determine how this potential new cytokine fits into the overall picture of TH1/TH2 immunoregulation.

A second P600 cDNA clone was isolated from another TH2 cDNA library, and the two clones were expressed by transfection into COS cells. The supernatants of such transfections contained a new 14-kDa protein, consistent with the length of the P600 open reading frame, assuming that the hydrophobic leader sequence was cleaved and a small amount of carbohydrate added. These transfection supernatants were tested in a variety of bioassays; in contrast to many T cell–derived cytokines, P600 appears to have a limited range of activities. The main activity discovered so far is the stimulation of a precursor cell in bone marrow to proliferate and differentiate into cells with macrophage-like properties.

The morphology of these cells is similar to the adherent cells that result from treatment of bone marrow with granulocyte macrophage colony-stimulating factor (GM-CSF) but distinct from the morphology of macrophages derived by growth of bone marrow cells in macrophage CSF (M-CSF). The precursor frequency of the P600-responsive cell is much lower than that of either the M-CSF– or GM-CSF–responsive precursors. Many of the P600-derived cells bear the MAC-1 and F4/80 surface antigens that are normally present on macrophages.

In functional assays the cells that result from growth in P600 are relatively well differentiated, as measured by their ability to cleave nitroblue tetrazolium much more effectively than GM-CSF– or M-CSF–derived cells. The P600-derived adherent cells also efficiently present alloantigens and soluble protein antigens to T cell clones, supporting their designation as a type of macrophage. However, the P600 cells show little or no phagocytosis of antibody-coated sheep erythrocytes, in contrast to the cells resulting from M-CSF treatment.

In general, TH1 cells produce cytokines that activate macrophages, whereas TH2 cells produce cytokines, such as IL-4 and IL-10, that inhibit macrophage cytotoxic functions. Thus it is not immediately apparent how P600, which appears to stimulate the growth of a particular type of macrophage, fits into the set of functions typical of TH2 cells. It is possible that P600 increases the number of macrophages without causing activation or that the P600-derived cells are a unique type of macrophage with an undiscovered role in the immune system or hemopoiesis. Studies are continuing on the nature of the P600-responsive cells and the effects of P600 treatment *in vivo*.

Dr. Mosmann is also Professor and Chair of the Department of Immunology at the University of Alberta, Edmonton.

CELLULAR AND MOLECULAR MECHANISMS OF VIRULENCE EXPRESSION IN *ENTAMOEBA HISTOLYTICA*

ESTHER OROZCO, PH.D., *International Research Scholar*

The long-range objective of Dr. Orozco's group is to understand the regulation of the molecular mechanisms involved in the expression of the virulence of *Entamoeba histolytica*, the protozoan parasite responsible for human amebiasis. They have described several molecules involved in the cytolytic activity of this intestinal parasite and are currently cloning and studying these molecules. Some are differentially expressed in pathogenic and nonpathogenic trophozoites. Dr. Orozco and her colleagues are focusing on the molecular basis of these differences. They also wish to determine the phyloge-

netic relationship between pathogenic and non-pathogenic *E. histolytica* strains.

The 112-kDa Adhesin of *Entamoeba histolytica*

Adherence, phagocytosis, and secretion are three key events in target cell destruction by *E. histolytica*. The exceptionally phagocytic and vesicle-rich trophozoites, the infective phase of this parasite, constitute an excellent model for studying these functions. Drs. Mario Alberto Rodriguez and Rossana Arroyo, in Dr. Orozco's group, identified and characterized *E. histolytica*'s 112-kDa adhesin, which is involved in phagocytosis and cytopathogenicity and plays an important role in the virulence of the parasite. It is absent or altered in nonpathogenic trophozoites and in virulence-deficient mutants. Recent experiments carried out by Dr. Christine Rigotier and Guillermina Garcia-Rivera showed that the adhesin is also involved in traffic and compartmentalization through vesicular intermediates en route to the plasma membrane to be secreted. Such an adhesin was not found in virulence-deficient mutants.

The 112-kDa adhesin has been partially purified by immunoaffinity chromatography using a monoclonal antibody. Two proteins of 70 and 55 kDa were also released from the immunoaffinity column. The three proteins were recognized by monospecific polyclonal antibodies against the 112-kDa adhesin. The adhesin, purified from preparative polyacrylamide gels, gave the same 70- and 55-kDa proteins when incubated in di-dithiothreitol.

Proteins of 112 and 70 kDa presented protease activity that was detected by their ability to degrade gelatin. A hypothesis is that the 112-kDa protein was broken down into these two peptides. Experiments are being performed to learn whether this mechanism occurs *in vivo* and whether it is related to the cytopathogenicity of the parasite.

Entamoeba histolytica Presents Variable Virulence, Selectively Virulent Antigens, and Genome Rearrangements through Axenization

Mechanisms involved in the expression of pathogenicity of *E. histolytica* were investigated through the process of axenization of a cloned nonpathogenic isolate, MAV-CINVESTAV, obtained from an asymptomatic carrier. Phenotypic characteristics traditionally related to the pathogenicity of this parasite, such as zymodeme, virulence, antigenic expression, presence of the 112-kDa adhesin and proteases, as well as the amount of DNA per cell, molecular karyotype, and organization of some virulence-involved genes, varied remarkably.

Cloned strain MAV-CINVESTAV (clone MAV-1), cultured under monoxenic and polyxenic conditions (MAVmx and MAVpx, respectively), presented nonpathogenic zymodeme and did not express virulence. However, trophozoites cultured under axenic conditions (MAVax) showed pathogenic zymodeme, a high rate of phagocytosis, and ability to damage target cells.

Virulence-involved antigens, such as the 112-kDa adhesin and the major cysteine protease, were expressed only in MAVax trophozoites. Striking changes in molecular karyotype, genome rearrangements, and gene amplification of virulence-related genes were also exhibited by the trophozoites through the axenization process in correlation with the expression of virulence *in vitro*. These results suggest that a cloned population of *E. histolytica* is able to modulate the expression of its virulence, probably through genomic rearrangements, including gene amplification, with the influence of medium conditions.

Genomic Variability in Closely Related Clones of *Entamoeba histolytica*

The molecular basis of the variability of *E. histolytica* was studied in clones A and L6, both obtained from the heterogeneous strain HM1-IMSS and, in clone C2, derived from clone A. By differential plaque hybridization of clones A and L6, the group isolated a 3.5-kbp cDNA clone, pMD. Certain pMD fragments recognized several transcripts in the three clones. However, another pMD fragment hybridized with a transcript of 0.63 kb was found only in clone A trophozoites. Southern blot analysis of both chromosomes and digested total DNA showed that all pMD fragments are encoded by a linked piece of DNA located in the 1.3- and 1.4-Mb chromosomes and in a 6-kbp EcoR1-EcoR1 DNA fragment.

Polymerase chain reaction (PCR) experiments demonstrated that the 0.63-kb differential transcript is absent or highly modified in the DNA of clones L6 and C2 trophozoites. The genetic relationship between the clones was verified by DNA polymorphism of several genes. Enzymatically digested DNA from clones A and C2 yielded almost identical restriction patterns, while differences between them and clone L6 were evident.

However, trophozoites of clone A cultured in various laboratories for several years gave different polymorphic fragments for many genes. MAV trophozoites did not hybridize with the differential pMD fragment, indicating that this sequence is absent or highly modified in them. These results indicated that *E. histolytica* presents a high genomic

plasticity that could explain, at least partially, the variability between pathogenic and nonpathogenic trophozoites.

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INTERACTIONS OF PROTEIN-TYROSINE KINASES WITH THEIR TARGETS

TONY PAWSON, PH.D., *International Research Scholar*

Many of the receptors for the polypeptide hormones that control cell growth, differentiation, and metabolism bind to cell surface receptors with protein-tyrosine kinase activity. A distinct class of tyrosine kinases, typified by the c-*src* gene product, are localized entirely within the cell and are implicated in a variety of signaling processes. In addition to their role in controlling the interactions of normal cells, constitutively active variants of tyrosine kinases are implicated in the development of some cancers. Dr. Pawson's laboratory is interested in the mechanisms by which tyrosine kinases activate intracellular signal transduction pathways and thereby regulate gene expression, cytoskeletal architecture, cell-cell interactions, and cell metabolism. The laboratory is also investigating the role of protein kinases and their targets in the control of vertebrate and invertebrate development.

Targets of Tyrosine Kinases Contain Src Homology 2 (SH2) Domains

A growing number of cytoplasmic signaling proteins contain small noncatalytic domains, termed SH2 and SH3. SH2 domains were originally identified in nonreceptor tyrosine kinases such as Src, Fps, and Abl, and were proposed to play a role in the interactions of these kinases with specific substrates. Subsequently, SH2 domains were detected in several signaling proteins that associate with, and are phosphorylated by, activated growth factor receptors, including Ras GTPase-activating protein (GAP), phospholipase C-γ (PLC-γ), and phosphatidylinositol (PI) 3'-kinase.

PI 3'-kinase is a heterodimer composed of an SH2-containing, receptor-binding subunit (p85), which apparently couples tyrosine kinases to the PI 3'-kinase catalytic subunit. The p85 regulatory subunit

of PI 3'-kinase is one of several SH2-containing proteins, including Crk, Nck, Shc, and Sem-5, that lack any obvious catalytic domains, and may therefore serve as adaptors to link receptor tyrosine kinases to specific signaling enzymes that have no intrinsic ability to interact with receptors.

The binding of cytoplasmic signaling proteins to activated receptor tyrosine kinases is mediated by their SH2 domains. Individual SH2 domains from proteins such as PLC-γ, GAP, Src, and PI 3'-kinase, synthesized in bacteria, bind to specific autophosphorylated growth factor receptors *in vitro*, although with different efficiencies. Hence a structurally and functionally diverse group of enzymes are endowed with the capacity to recognize activated receptors with high affinity by virtue of their common SH2 domains.

A variety of data indicate that SH2 domains bind directly to specific tyrosine-phosphorylated sites located within noncatalytic regions of activated receptors. As an example, the kinase-insert region of the macrophage colony-stimulating factor (CSF-1) receptor, although not essential for receptor kinase activity, is both necessary and sufficient for binding to PI 3'-kinase, provided that the insert is tyrosine-phosphorylated. Although the kinase insert of the mouse CSF-1 receptor becomes autophosphorylated at multiple tyrosine residues following CSF-1 stimulation, binding of the SH2 domains of the p85 PI 3'-kinase subunit requires phosphorylation of Tyr[721], which lies within a consensus sequence for PI 3'-kinase association and is entirely independent of autophosphorylation at other sites.

Such results indicate that SH2 domains recognize phosphotyrosine but require a specific surrounding amino acid sequence for high-affinity binding. Mutagenesis of the GAP SH2 domains has implicated an

invariant arginine as crucial for binding to activated growth factor receptors and other phosphotyrosine-containing proteins. This arginine may therefore be involved in the direct recognition of phosphotyrosine. The elements within SH2 domains that confer their specificity for different phosphorylated sites are of the greatest interest.

A Novel SH2-containing Protein Implicated in Mitogenic Signal Transduction

The SH2-mediated association of signaling proteins such as PLC-γ and PI 3'-kinase with autophosphorylated receptors appears important for their activation in response to growth factor stimulation. This suggests that SH2 domains are likely to be a hallmark of many tyrosine kinase targets and hence that the isolation of novel SH2-containing proteins may yield new information concerning the regulation of intracellular signal transduction in response to growth factors.

In collaboration with Dr. Pier Giuseppe Pelicci, Dr. Jane McGlade has identified the *SHC* gene products, which contain a single SH2 domain and a more amino-terminal glycine/proline-rich region. *SHC* overexpression induces growth factor–independent proliferation and malignant transformation of fibroblasts. Shc proteins become highly tyrosine-phosphorylated following stimulation with a variety of growth factors and, through their SH2 domain, become physically associated with activated growth factor receptors. In addition, the *SHC* gene products appear to be prominent substrates of the v-Src, v-Fps, and Bcr-Abl oncoproteins. These results suggest that Shc proteins may be adaptors that couple a wide spectrum of receptor and nonreceptor tyrosine kinases to a signaling pathway that controls proliferation. The tyrosine phosphorylation of Shc proteins induces their association with a 23-kDa polypeptide, which is a good candidate for the downstream Shc target.

It is evident that there are SH2-containing gene products, in addition to *SHC*, that remain to be described. A novel, widely expressed tyrosine phosphatase with two SH2 domains has recently been isolated and is being characterized.

A Network of SH2-mediated Interactions in Signal Transduction

The role of SH2 domains in directing the association of signaling proteins with growth factor receptors has been extensively explored. SH2 domains also appear important in the subsequent interactions of proteins such as GAP and Src with cytoplas-

mic tyrosine-phosphorylated proteins. In particular, the Src SH2 domain may play multiple roles in regulating c-Src tyrosine kinase activity and in substrate recognition. Notably, the SH2 domain of the oncogenic Src tyrosine kinase associates with a specific subset of the proteins that are phosphorylated by the Src kinase domain. These interactions may serve several functions, including protecting these substrates from dephosphorylation and promoting the formation of signaling complexes.

Biological Functions of Receptor Tyrosine Kinases and Their Targets

The mechanisms controlling the interactions of tyrosine kinases with cytoplasmic signaling proteins, and the functions of specific signal transduction pathways in regulating cell phenotype, are being variously investigated. At a molecular level, the biophysical and biochemical properties of SH2-phosphoprotein complexes are being pursued. The effects of specific SH2-containing proteins on cellular responses to extracellular signals are under investigation. In addition, genetic approaches involving *Drosophila melanogaster*, *Caenorhabditis elegans*, and the mouse are being pursued to define the role of these gene products in development.

Although a plethora of receptor tyrosine kinases have been identified, the biological functions of most of these are unknown. Dr. Pawson's laboratory previously described a mammalian receptor tyrosine kinase, encoded by the *elk* gene, that is specifically expressed in the brain. More recently Dr. Mark Henkemeyer demonstrated that a novel receptor tyrosine kinase of the *eph/elk* family, termed *nuk*, is segmentally expressed during the early development of the mouse central nervous system and may therefore be involved in patterning of the nervous system. The function of this gene and its product is under investigation, using both genetic and biochemical approaches.

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CHEMICAL AND FUNCTIONAL CHARACTERIZATION OF SCORPION TOXINS

LOURIVAL DOMINGOS POSSANI, PH.D., *International Research Scholar*

Scorpionism is a public health problem in Mexico. More than 200,000 people are stung by scorpions annually, and about 700 die. For 15 years Dr. Possani and his colleagues have been studying the small peptides responsible for the lethality of scorpion stings.

Three distinct families of peptides have been isolated and characterized from the venom of these arachnids: 1) short-chain peptides of about 38–39 amino acid residues, blockers of K$^+$ channels in various excitable tissues; 2) medium-chain peptides (61–65 amino acid residues), blockers of Na$^+$ channels in mammalian tissues; and 3) long-chain peptides (about 70 amino acid residues), blockers of Na$^+$ channels of tissues in crustaceans and insects.

The research conducted in the past year was focused on the following aspects: further characterization of Na$^+$ channel–blocking peptides, isolated from the venom of the scorpion *Centruroides noxius*; primary structure determination of newly purified K$^+$ channel toxins; and cloning of scorpion toxin genes. Additional work was performed on the pancreatic secretagogue effect of toxins purified from scorpion venoms, in collaboration with Dr. Paul Fletcher (East Carolina University, Greenville, North Carolina).

Definition of Four Distinct Epitopes in the Structure of Toxins from the Genus *Centruroides*

Toxins 2 and 3, isolated from the venom of the Mexican scorpion *Centruroides noxius* Hoffmann, were purified and their amino acid sequences determined (66 amino acid residues each). Sequence comparison indicates 79% identity in the primary structure of both toxins and shows a high similarity to previously characterized *Centruroides* toxins, the most similar toxins being toxin 1 from *Centruroides limpidus tecomanus* and toxin 2 from *Centruroides suffusus*. Six monoclonal antibodies

(mABs) were obtained and used to characterize these toxins immunochemically. Four different mABs reacted only with toxin 2, whereas the two other mABs reacted with both toxins 2 and 3 with the same affinity.

Simultaneous binding of mAB pairs to the toxin and cross-reactivity of the venoms of different scorpions with the mABs were examined, allowing the definition of four distinct epitopes (A–D) in the structure of toxins from the genus *Centruroides*. Epitope A is topographically unrelated to epitopes B, C, and D, but the latter three appear to be more related or in close proximity to one another. Epitope A was found in all *Centruroides* venoms tested (*C. noxius, C. elegans, C. suffusus, C. l. limpidus, C. l. tecomanus,* and *C. l. acatlanensis*), as well as in four different purified toxins of *C. noxius*, and thus seems to correspond to a highly conserved structure. All six mABs inhibited the binding of toxin 2 to rat brain synaptosomal membranes, but only the BCF2 mAB, which belongs to the IgG$_{2a}$ subclass, displayed a clear neutralizing activity *in vivo*.

New K$^+$ Channel–blocking Toxins Purified from the Venom of *C. l. limpidus*

Several years ago Dr. Possani's group discovered noxiustoxin (NTX), the first peptide purified from scorpion venom for which a clear effect on the blockade of K$^+$ channels was shown. Presently the research effort is being focused on the isolation and characterization of similar peptides from other scorpion species. In this respect, a set of different clones producing mABs against NTX was obtained. Using an ELISA assay with these mABs, two new toxins were purified and sequenced from the venom of the Mexican scorpion *C. l. limpidus*. A series of distinct chromatographic fractions from this venom were attached to the plates and developed with the mAB anti-NTX. With this technique two different peptides were purified and sequenced, in collaboration

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with Dr. Brian Martin (National Institute of Mental Health).

Comparison of the primary structures showed important similarities among these peptides and with that of NTX and charybdotoxin. Functional characterization of the newly purified peptides was carried out by binding displacement experiments using rat brain synaptosomes and radiolabeled NTX. Both toxins from *C. l. limpidus* displace 50% of membrane-bound NTX at the level of 100 pM. Further experiments conducted with cerebellum granule cells, using the patch-clamp whole-cell configuration technique, showed that these peptides are capable of blocking one type of K^+ channel present in this preparation.

Cloning of Scorpion Toxin Genes from *Centruroides* Venomous Glands

A cDNA library was prepared from venomous glands of *C. noxius* scorpions. The λgt11 library was probed with specific oligonucleotides chosen from known amino acid sequences of Na^+ channel–blocking toxins from scorpions of the genus *Centruroides*. Among several positive clones carrying toxin genes, four were selected and their nucleotide sequences determined. A comparative analysis of these sequences with one of reported scorpion toxins revealed that these cDNAs code for a family of very similar toxins. One of the clones corresponded to toxin II-10 of *C. noxius*; the others are closely related to toxin 1 from *C. noxius* and variant 3 from *C. sculpturatus*. Southern blot genomic analysis showed a minimum size of ∼600 bp as an *Eco*R1 fragment for elements of this family.

Polymerase chain reaction (PCR) amplifications of *C. noxius* genomic DNA, and hybridization of PCR products with specific probes, indicated that the genomic structural regions that code for the toxin-cloned genes do not contain introns. Comparison of these nucleotide sequences with those of cDNAs that code for North African scorpion toxins revealed that they share common features. It seems that this family of genes evolved from an ancestral gene that has experienced several duplications, and each duplicated gene has evolved independently by means of insertions, deletions, and point mutations.

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PROTEIN CRYSTALLOGRAPHY IN THE STUDY OF INFECTIOUS DISEASES

RANDY J. READ, PH.D., *International Research Scholar*

The research in Dr. Read's laboratory is aimed at advancing the understanding of infectious diseases at the molecular level, using information from the three-dimensional structures of key proteins. There are two major aspects to this work. First, the x-ray crystal structures of a number of proteins involved in pathogenesis are being determined. Second, computational techniques are being developed to exploit such structural information in the rational design of new drugs for the treatment of infectious diseases.

Bacterial Toxins

Many pathogenic bacteria produce toxins that cause cell and tissue damage and can be responsible for the most severe effects of the illness. Bacterial toxins often have an A-B structure in which the B (binding) subunit binds to the surface of a target cell and the A (active) subunit enters the cell, carrying out the toxic action. Two such toxins are being studied: verotoxin-1 (with Dr. James Brunton, University of Toronto) and pertussis toxin (with Dr. Glen Armstrong, University of Alberta, and with Connaught Laboratories in Toronto).

The Shiga toxin family is a group of closely related toxins, including Shiga toxin, produced by *Shigella dysenteriae* type 1, and verotoxins (VTs), produced by certain strains of *Escherichia coli*. Shiga toxin is associated with bacterial dysentery, a serious problem in many developing countries. The strains of *E. coli* that produce VTs cause a disease often referred to as "hamburger disease," because it can be acquired from contaminated hamburger. VTs can provoke the hemolytic uremic syndrome and are

thereby the major cause of acute kidney failure in children.

The structure of the B subunit of VT-1, a member of the Shiga toxin family, has been determined by multiple isomorphous replacement (MIR). The B subunit forms a pentamer that binds to the carbohydrate component of a cell-surface glycolipid, globotriaosylceramide (Gb_3). Comparison of the amino acid sequences of all the toxins in this family indicates that a surface cleft between B-subunit monomers is likely to be the carbohydrate-binding site. Crystallographic binding studies are under way to test this prediction.

The VT-1 B subunit bears a striking resemblance to the B subunit of the *E. coli* heat-labile enterotoxin (LT), a member of the cholera toxin family. This is unexpected, because the associated A subunits are completely unrelated, the B subunits are very different in size (69 residues for VT-1 vs. 103 for LT), and there is no detectable sequence homology. In collaboration with Drs. Titia Sixma and Wim Hol (University of Groningen), the two B subunits have been compared. Fifty-two amino acids superimpose very closely in the structural alignment, yet only three are identical.

Pertussis toxin (PT) is produced by *Bordetella pertussis*, the bacterium that causes whooping cough. This toxin's role in improved vaccines is a major interest. Killed whole-cell vaccines are reasonably effective but have an undesirable level of toxicity that could be reduced or eliminated in a defined vaccine produced from genetically engineered proteins. PT has been shown to be a necessary component of effective whooping cough vaccines. A three-dimensional structure would help show how to remove its toxic activities while preserving the antigenic determinants necessary to evoke protective immunity. In addition, it would lead to a more complete understanding of the biochemistry of PT action. Considerable progress has been made on this structure. MIR, using synchrotron data collected at the Photon Factory in Japan, has given useful phase information. This has been improved by a combination of electron density-averaging and solvent-flattening techniques.

Pseudomonas aeruginosa Pilin

Pseudomonas aeruginosa is an opportunistic pathogen that infects burn victims and immunocompromised patients. It is also one of the major pathogens infecting the lungs of cystic fibrosis patients. Colonization of epithelial cell surfaces is promoted by pili, filaments that are formed from a helical array of identical pilin subunits. PAK pilin

(from *Pseudomonas aeruginosa*, strain K), obtained in collaboration with Dr. William Paranchych (University of Alberta), has been crystallized. Problems with crystal quality that have hindered progress are being overcome by improving the purity of protein preparations. The three-dimensional structure would aid in understanding the details of cell-surface binding, which could be used to devise strategies to interfere with colonization.

Fab (antigen-binding fragment of immunoglobulin) fragments from two monoclonal antibodies raised against PAK pilin have been crystallized (with Dr. Randall Irvin, University of Alberta). Pilin-Fab complexes can be formed in solution, and attempts to crystallize them are under way. In addition, a 17-residue peptide from the carboxyl terminus of PAK pilin, obtained from Dr. Robert Hodges (University of Alberta), has been shown to bind to one of these antibodies, PK99H. Attempts to crystallize a complex with this peptide are in progress. The structures of antibody complexes could be relevant to the development of peptide vaccines, since the monoclonal antibodies have been shown effective in passive immunization studies.

Computer-aided Drug Design

Most drugs with known mechanisms of action bind specifically to a drug receptor, often a protein molecule. Since crystallography allows one to examine the structures of receptors and the details of their interactions with drugs, it has the promise of helping to improve existing drugs, or even to invent new ones. Progress has been made on improving existing drugs using structural information, but the problem of exploiting such information to design new drugs from scratch is still extremely difficult. There are probably billions of compounds that might be used as drugs; determining which of these might bind to the target protein is far from trivial.

A "divide and conquer" approach to drug design should reduce the magnitude of this problem. The vast set of possible compounds is made up of various combinations of a much smaller set of molecular fragments. It is proposed that drugs can be designed by using a computer first to dock members of a library of fragments to the region of the desired binding site, then to combine docked fragments to form chemically sensible molecules. Some success in the fragment-docking problem has been achieved by using a Monte Carlo procedure to find favorable interactions. Multiple random docking trials are performed to ensure that most of the favorable interaction sites will be found. In test calculations,

known complexes have been reassembled, with the correct interaction consistently being at or near the top of the list. Fragments of known ligands can also be docked. Current work is aimed at incorporating recent advances in the calculation of interaction energies and at developing the fragment-matching algorithms. As energy calculations improve and computers become faster and cheaper, this method should become a valuable drug design tool.

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REPRESENTATION OF TACTILE SIGNALS IN THE SOMATIC SENSORY CORTEX

Ranulfo Romo, M.D., Ph.D., *International Research Scholar*

The somatic sensory system of subhuman primates seems an appropriate model for approaching the question of how tactile signals are represented in the cerebral cortex. The hands of these animals and their brain structures related to somatic sensibility are similar to those of humans. Similar sensory performance in somatesthetic tasks has also been observed in both primates. Moreover, the exploratory movements of the hands have similar characteristics, since the somatic and motor systems in both cases are anatomically linked.

Dr. Romo's laboratory is involved in studying the representation of tactile signals in the cerebral cortex and the mechanisms by which these signals are processed by the motor centers to guide motor behavior. A first objective in this part of the research program is to define quantitatively the neural representation of moving tactile signals in areas 3b and 1 of the primary somatic sensory cortex. The study consists of recording single neurons of areas 3b and 1 with receptive fields in the primate's hand. These receptive fields are scanned with a probe in different directions and at variable speeds, at fixed traverse distance and constant force. With these data, Dr. Romo and his colleagues have defined the speed encoded by each neuron and the direction of the moving tactile stimulus.

A Tactile Stimulator for Studying Motion Processing in the Somatic Sensory System of Primates

It is well known that neurons in areas 3b and 1 of the somatic sensory cortex possess properties and receptive fields similar to those found in cutaneous primary afferents. However, the dynamic responses of these neurons to tactile stimuli are poorly understood. A difficulty encountered in somatosensory research has been the control of the presentation of stimulus parameters. For this purpose, Dr. Romo's laboratory has built a tactile stimulator for automatic presentation of very well controlled, moving stimuli in the receptive fields of the recorded neuron. This apparatus is used to assess the responses of neurons of the somatic sensory system to stimuli moving in any traverse distance (range, 2–20 mm), with a variety of velocities (range, 4–120 mm/s) and forces (range 0–60 gf), and in any scanning direction. The stimulator is highly automated and is currently used in human psychophysics and in combined psychophysical and neurophysiological studies in behaving monkeys.

Responses of Single Neurons of Areas 3b and 1 to the Speed of the Moving Tactile Stimulus

Dr. Romo and his colleagues have studied quantitatively 178 neurons in five hemispheres of three awake *Macaca mulatta*s. Ninety-six of these neurons were located in area 1, and 82 in area 3b, all anatomically identified. According to their submodality (defined by their temporal adaptation to a steady, light mechanical stimulus applied to their receptive field), 88 were classified as rapidly adapting (RA; 61 in area 1, and 27 in area 3b), 61 as slowly adapting (SA; 19 in area 1, and 42 in area 3b), 3 as pacinian (PC; all recorded in area 1), and 26 as presenting RA-SA properties (13 in area 1 and the same number in area 3b).

The distribution of submodalities encountered in this study agrees with that in the literature. The firing rate of 129 neurons (72%) of the 178 studied responded at velocities from 20 to 100 mm/s. Groups of neurons of areas 3b and 1 were tuned to low (20 mm/s), intermediate (50 mm/s), or high

velocities (100 mm/s), or broadly tuned (20–100 mm/s). No differences were encountered between areas and submodalities, suggesting that both SA and RA neurons in the somatic sensory cortex encode the speed of the tactile stimulus. Psychophysical experiments in humans and monkeys are in progress to determine the capacity to detect and discriminate velocities, and whether the responses of somatic neurons encountered in the study account for this property.

Responses of Single Neurons of Areas 3b and 1 to the Direction of Movement Across the Primate's Hand

An analysis of variance was performed to identify the neurons whose discharge rate varied significantly with the direction of a stimulus across the receptive field, in the skin of the primate's hand (ANOVA, F test, $p < 0.05$). It was found that ~74% of the 178 tested neurons responded significantly to a certain direction of the movement across the receptive field. The distribution of the number of neurons that responded to the direction of movement is similar in areas 3b and 1. A multiple regression model was used to determine whether the discharge rate varied with the direction of movement. Sixty-seven (32 in area 1; 35 in area 3b) neurons showed

good fits to the model ($p < 0.05$; $r > 0.7$). This directional tuning develops as a function of speed. Higher directional tuning is found at intermediate speeds (20–50 mm/s), and lower below and above this range.

These observations point out that neurons (30%) in areas 3b and 1 of the somatosensory cortex of awake monkeys possess directional tuning functions. In other words, the discharge rate is a function of the difference between the preferent angle and the direction of the stimulus. There is a relationship between the directional properties and the speed of the stimulus, since a large percentage of neurons show directional tuning to speeds in the range of 20–50 mm/s. It is predicted from these results that the signaling of the direction of the stimulus is carried out by a neuronal population distributed in the somatosensory cortex in the form of a neuronal population vector. Experiments are in progress to determine the discriminative properties of neurons of the somatic sensory cortex as the animal discriminates the angle of the direction of movement.

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THE GENETICS AND EMBRYOLOGY OF DEVELOPMENT

JANET ROSSANT, PH.D., *International Research Scholar*

The aim of research in Dr. Rossant's laboratory is to understand the development of early cell lineages in the mouse embryo, using embryological and genetic manipulation. Development of the trophectoderm lineage of the blastocyst, and mechanisms of anterior-posterior patterning of the early neural ectoderm, are current areas of interest.

The Role of Mesoderm-Ectoderm Interactions in Neural Patterning

In other vertebrate embryos, such as *Xenopus* and chick, it is known that signals pass from the underlying mesoderm to initiate neural induction in the ectoderm of the gastrula stage. Regionalization along the body axis also involves regionalized inducing signals from the mesoderm. There is little evidence on the nature of such interactions in the mouse embryo. An *in vitro* explant-recombination system has been established for the postimplantation mouse embryo, in which pieces of germ layers can be

grown in isolation or in different combinations. With this system, it has been shown that markers of mid-hindbrain development, the *Engrailed-1* and *-2* genes, are induced by underlying mesoderm at the late-primitive-streak/early-head-fold stage. Only anterior and not posterior mesoderm possesses inducing ability, but posterior and anterior ectoderm are capable of responding. Preliminary data suggest that the events leading to the expression of other regionalized markers in the brain also occur around the head-fold stage of development.

The Role of Retinoic Acid in Anterior-Posterior Patterning

One factor that may be involved in anterior-posterior patterning at the gastrulation and neural-plate stages of development is retinoic acid (RA) (or other retinoids). There is considerable circumstantial evidence for this, based largely on the teratogenic effects of excess RA and the presence of RA

receptors and binding proteins in the early embryo. Transgenic mice carrying a response element for RA (RARE) in front of a neutral promoter-*lacZ* construct have provided *in vivo* evidence that there is an active RA transcriptional response in the embryo. The transgene shows no expression until the late-gastrula/early-neural-plate stage and is then expressed only in the posterior half of the embryo. As development proceeds the anterior boundary of transgene expression regresses in concert with the establishment of the anterior boundaries of 3′ members of the *Hox-2* gene family, believed to be important in defining regional identity along the body axis.

Further evidence linking RA and *Hox* gene expression comes from studies on the effects of RA treatment *in vivo* on *Hox* gene expression in embryos. Treatment of gastrulating mouse embryos with excess RA causes an anterior shift of *Hox-2.9* and *Hox-2.8* expression within four hours of treatment. More-posterior (5′) *Hox-2* genes are not affected. The response of the *Hox-2* genes at different stages is complex, but the general picture seems to be that anterior (3′) *Hox-2* genes whose boundaries end up in the hindbrain or beginning of the spinal cord can be shifted more anteriorly by RA in the neural tube until they reach their full anterior extent of expression. After this they are resistant to RA treatment. Combined with the known teratogenic effects of RA on hindbrain and *in vitro* evidence from other laboratories of differential sensitivity of *Hox* genes to RA along the 3′-to-5′ direction of the cluster, this suggests that RA may be a modulator of the boundaries of *Hox* gene expression. The source of RA in the embryo is still unknown but may be the node at the anterior end of the primitive streak that first appears concomitant with the onset of the RARE *lacZ* transgene expression.

Gene Trap Screen in ES Cells

In collaboration with Dr. Alexandra Joyner (HHMI, International Research Scholar), a large-scale screen is being performed for new genes expressed in spatially restricted domains around gastrulation, using gene-trap approaches. The gene trap vector contains the *Escherichia coli lacZ* gene as a reporter with a splice acceptor but no promoter up-stream. Insertion of this vector into a gene in the right orientation and reading frame will disrupt the host gene and allow a fusion transcript and protein to be produced between the host gene and *lacZ*. After introduction of this vector into embryonic stem cells, clones selected for *lacZ* expression are injected into blastocysts. Sets of chimeras from individual clones are screened for *lacZ* expression at gastrulation. Those showing a pattern of interest can be taken through the germline to study the possible mutant phenotype, and exons in the host gene can be cloned from *lacZ*-containing cDNAs. This is thus an efficient means of identifying and mutating mouse developmentally regulated genes. A screen of 300 such lines for expression in chimeras is almost complete, and a number of interesting patterns have been observed. One line of particular interest for possible involvement in anterior-posterior patterning shows *lacZ* expression in the node and head process derivatives. Cloning and characterization of this gene is under way.

Dr. Rossant is a Senior Scientist at the Samuel Lunenfeld Research Institute of Mount Sinai Hospital, Toronto, and Professor of Molecular and Medical Genetics at the University of Toronto.

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CONTRIBUTION OF AREA MST TO THE SPATIAL FUNCTIONS OF THE PARIETAL LOBE

Jean-Pierre Roy, M.D., *International Research Scholar*

Dr. Roy studies the response of neurons in the medial superior temporal (MST) area of the cerebral cortex of the behaving Rhesus monkey. The goal of this research is to understand how low-level signals, such as those indicating the direction of motion or binocular disparity preferred by a neuron, are transformed into higher-level signals—for example, those defining the spatial structure of the environment.

During a fellowship with Dr. Robert Wurtz at the National Institutes of Health, Dr. Roy found a subclass of cells in area MST that responded for one disparity when the stimulus moved in one direction —for example, crossed disparities corresponding to motion in front of the point fixated—and responded for the opposite disparity, here uncrossed disparities, when the stimulus moved in the opposite direction, corresponding to motion behind the point fixated. These cells, the disparity-dependent direction-selective (DDD) neurons, were proposed to be involved in transforming the visual information about the foreground and background motions into information about the direction of self-motion of the subject in his or her environment.

This can be understood if one pictures what happens to the images of the environment as one moves forward while looking at an object located roughly perpendicular to the direction of motion. The foreground, the environment in front of the object fixated, appears to move in one direction, and the background, the environment behind the object fixated, moves in the opposite direction. A DDD neuron responding to leftward motion of the foreground and rightward motion of the background will respond for rightward motion of a subject looking forward irrespective of where in depth the subject looks. This neuron, by combining the two correct signals about disparity and direction, indicates the direction of self-motion relative to the object fixated.

These neurons provide a way of asking an important question about the transformation of another type of low-level signal, relative speeds, into a signal about the spatial structure of the environment. Because the condition under which the DDD neuron discharges is known (self-motion in one direction with perpendicular gaze), it is possible to explore this second question. When moving in one direction while looking perpendicularly, a parameter different from direction and disparity has to be examined—relative speed. Objects from the environment that are close to the point fixated, whether they are in the foreground or background, move relatively slowly. Objects that are far from the object, i.e., very far or very close, move relatively fast. Relative speed contains information about the depths of objects with respect to the object fixated. It has to be understood that it is not absolute speed that carries the depth information, since the absolute speeds will vary with the speed of displacement of the subject. Rather, it is the speed ratios between objects at different depths, i.e., speed gradients.

To explore the response of DDD neurons from MST to such speed gradients, Dr. Roy is comparing the response of one DDD neuron with stimuli that do and do not include a speed gradient. This response is also compared with stimuli that do and do not include a disparity gradient to evaluate the contribution of this parameter. The prediction is that speed gradient does contribute to the cell's response, and if so, this could be how a DDD neuron conveys information about the three-dimensional structure of the environment.

Preliminary data obtained recently seem to confirm this prediction, but more work remains to be done before any firm conclusion can be reached.

Dr. Roy is Assistant Professor of Neurology and Neurosurgery at the Montreal Neurological Institute, McGill University.

MOLECULAR PROPERTIES OF NEURONAL VOLTAGE-GATED CALCIUM CHANNELS

Terry P. Snutch, Ph.D., *International Research Scholar*

Dr. Snutch's laboratory investigates signal transduction in the mammalian central nervous system (CNS). The entry of calcium into neurons directly affects membrane potential and contributes to the electrical properties of cells. In addition, calcium acts as a second messenger and further affects cellular events by regulating the activity of calcium-dependent enzymes and ion channels. The rapid entry of calcium into excitable cells is mediated by specific voltage-gated channels. The long-term goals of the research are to understand the molecular nature of the diverse roles that these channels play in such processes as neuronal firing patterns, neurotransmitter release, neuromodulation, and gene regulation.

Diversity of Neuronal Calcium Channels

In both neurons and other cell types, various calcium channels can be distinguished by their electrophysiological and pharmacological properties (designated T, L, N, and P types). A major concern is defining the molecular nature of calcium channel diversity. Dr. Snutch's laboratory has proposed that the expression of distinct isoforms of the pore-forming α_1 subunit of calcium channels is responsible for a large amount of the observed functional heterogeneity. Recent molecular cloning studies in the laboratory have established that at least four distinct subtypes of calcium channel α_1 subunits are expressed in the rat CNS (designated rbA-I, rbB-I, rbC-I, and rbD-I). The four cloned types of these subunits are between 240 and 262 kDa in predicted molecular mass, and each possesses four internal repeated domains (I–IV) that are modeled to contain six to eight transmembrane regions. The overall transmembrane topologies and predicted secondary structures are similar to those proposed for voltage-gated sodium and potassium channels and indicate a common evolutionary origin for these molecules.

Although the four calcium channel α_1 subunits of brain are highly similar in the four repeated domains, they diverge significantly from one another in both the segment-linking domains II and III and in their carboxyl regions. Both of these regions are modeled to be cytoplasmic. They contain many consensus sites for modulation by cAMP- and cGMP-dependent kinases, protein kinase C, and calmodulin kinase II, and may account for differences in the modulation of neuronal calcium currents. Examination of the positively charged S4 segments shows that two distinct patterns occur in this putative voltage-sensor region. In domains III and IV, the rbC-I and rbD-I channels have identical S4 segments, whereas the rbA-I and rbB-I channels have different patterns of positively charged residues in both domains. Structure-function studies will determine how these differences contribute to the diverse activation properties observed for calcium channels.

To determine the relationship between the cloned α_1 subunits and calcium channels defined in neurons, Dr. Snutch, in collaboration with Dr. William Catterall (University of Washington), generated polyclonal antisera specific for the four classes of brain calcium channels. Immunoprecipitation of rat brain membranes radiolabeled with calcium channel antagonists gave the unexpected result that both the rbC-I and rbD-I α_1 subunits encode distinct L-type calcium channels. Similar experiments show that the rbA-I α_1 subunit encodes neither L- nor N-type calcium channels. Together with the high levels of expression of rbA-I observed in the cerebellar Purkinje cells, these results suggest that rbA-I may encode a P-type calcium channel. These studies also demonstrate that rbB-I encodes an ω-conotoxin–sensitive N-type calcium channel. The molecular cloning of an N-type channel is significant in that these channels are thought to be concentrated at presynaptic nerve termini and that they play a major role in mediating chemical synaptic transmission.

The availability of probes for an N-type channel will aid in studies examining the physiological roles of this channel in neuromodulation and in the small-cell lung carcinoma associated with Lambert-Eaton myasthenic syndrome. In agreement with studies indicating that N-type calcium channels are a heteroligomeric complex, the expression of the rbB-I polypeptide alone does not result in functional N-type calcium channels. The goal of this project now is to express the cloned α_1 subunits together with ancillary calcium channel subunits and to determine their electrophysiological and pharmacological properties.

Alternative Splicing of Calcium Channels

Although initial studies demonstrated the existence of the four main classes of brain calcium channel α_1 subunit, further analysis shows that heterogeneity also exists within each class. Molecular cloning and polymerase chain reaction studies demonstrate that the rat genome encodes single genes for the rbC and rbD α_1 subunits and that isoforms are generated by mutually exclusive alternative splic-

ing events. Of potential functional significance is the fact that the isoforms found within the rbC and rbD classes are often the result of the nearly precise substitution of putative transmembrane segments. Isoforms for the rbA and rbB α_1 subunits have also been detected, although most of the divergence, in contrast to the rbC and rbD channels, resides in the carboxyl segments of the polypeptides. This unanticipated degree of calcium channel heterogeneity may account for variations in the electrophysiological and pharmacological properties of calcium channels described in different cell types.

Differential Expression of Calcium Channels

The distribution of calcium channels in the mammalian CNS has been mostly defined by studies utilizing autoradiographic localization of radiolabeled antagonists. The availability of gene-specific probes allows more-direct analysis of calcium channel gene expression. At the regional level, all of the cloned calcium channel α_1 subunits are expressed through-

out the CNS. However, the expression of some isoforms generated by alternative splicing is spatially regulated. In addition, *in situ* hybridization studies demonstrate that, at the cellular level, the various calcium channel isoforms are preferentially expressed in subsets of neurons. For example, in the cerebellum, rbA-I transcripts are detected at 5- to 10-fold higher levels in Purkinje cells compared with other cell types. Given the complex morphology of neurons, it is likely that both the spatial distribution and the density of calcium channel subtypes contribute to the integration of neuronal excitability. This is being addressed utilizing polyclonal antisera to define the subcellular localization of the various subtypes of calcium channels in neurons.

Dr. Snutch is Assistant Professor in the Biotechnology Laboratory and a member of the Department of Zoology and Division of Neuroscience at the University of British Columbia, Vancouver.

STUDIES IN MOLECULAR GENETICS

Lap-Chee Tsui, Ph.D., *International Research Scholar*

Molecular Genetics of Cystic Fibrosis

To investigate the basic defect in cystic fibrosis (CF), Dr. Tsui and his colleagues continue to search for mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene. The large size of the gene, with 27 exons spanning 230 kb of DNA, has rendered the detection of microscopic mutations difficult. Furthermore, because of the lack of a convenient functional assay for CFTR, it is not easy to distinguish a truly disease-causing mutation from a benign amino acid substitution. Nevertheless, an international consortium has been formed to collect the data, and more than 200 putative mutations and 100 sequence variations have already been identified. Dr. Tsui's laboratory has played a central role in the consortium, through active compilation and dissemination of gene sequences and mutation data.

The consortium data show that ΔF508 is still the most frequent mutation, accounting for an overall 67% of the world CF mutant alleles. The remaining mutations are mostly rare and highly heterogeneous among different populations. After testing more than 80 different mutations in a population of more than 500 patients attending the CF clinic at the Hospital for Sick Children, Toronto, it has been possible

to define the mutations in only 85% of the mutant chromosomes. The same level of coverage has been observed for most other populations. Therefore these results strongly suggest that direct DNA testing of mutations should be done in combination with closely linked, highly informative DNA markers for genetic diagnosis in families with CF.

The varied degree of severity of the disease among different CF patients suggested that the phenotypes could be conferred, at least in part, by the genotypes at CFTR. Studies in this laboratory demonstrated that patients homozygous for the ΔF508 mutation were almost exclusively pancreatic-insufficient (PI), and there was an excess of pancreatic-sufficient (PS) patients with non-ΔF508 mutations. It has been Dr. Tsui's hypothesis that patients who are homozygous or compound-heterozygous of severe mutations, which include ΔF508, are expected to be PI; in contrast, patients with one or two copies of the other class (i.e., mild) of alleles are expected to be PS. Based on the hypothesis, ~90% of the mutant alleles are expected to be severe and 10% mild. Through further investigation it has been possible to classify most of the mutations detected in the patient cohort at the Toronto clinic into the two

groups. Genotype association with other clinical manifestations, such as lung and liver disease, are also being investigated, but no genotype correlation is immediately apparent.

A full-length cDNA for CFTR was constructed and inserted into several expression vectors to facilitate direct biochemical and physiological analysis. Results of DNA transfection studies showed that this cDNA could confer a cAMP-regulated chloride channel activity *de novo*, suggesting that CFTR is a chloride channel itself. Although most published studies involved the use of a transient assay, the expression system developed in Dr. Tsui's laboratory required the establishment of permanent transformed cell lines so that more subtle alteration of channel properties could be detected. Preliminary data from the analysis of constructs reproducing some of the naturally occurring mutations show that they are in good agreement with data predicted from the severity of pancreatic involvement.

Multiple transcription initiation sites, alternative splicing, and trans-splicing of CFTR transcripts were documented for the CFTR gene in different tissues, but a major initiation site could be identified within a short distance downstream from an undermethylated CpG-rich region. Deletion and transfection studies showed that the basal promoter element for the CFTR gene was within 250 bp of the major transcription initiation site. In addition, a negative regulatory element could be located immediately upstream of this sequence, and proper expression of CFTR *in vivo* might require additional cis-regulatory element(s) yet to be identified.

Experiments are also in progress to exploit the yeast *STE6* gene as a genetic system to gain some insight into the structure and function of the first ATP-binding domain (NBF1) in CFTR. Through replacement of the structurally similar domain in *STE6* with CFTR sequences, hybrid expression vectors have been constructed whereby mutations in CFTR could be measured by efficiency of yeast mating, the normal function of *STE6*. In addition, second-site mutations that could rescue the ΔF508 mutation have been found in adjacent regions of the deletion. A systematic survey of these second-site mutations should provide important information about the structure of NBF1 and the possibility of its application in drug design.

Lastly, to generate an animal model for the study of CF, work has been initiated to inactivate the mouse Cftr gene via homologous recombination in embryonic stem cells. The attempt to interrupt exon 10 has so far been unsuccessful, and the current targets in this laboratory are exons 1 and 13. The availability of a mutant mouse strain should greatly facilitate studies to understand the pathophysiology of CF and improve means of treatment for the disease.

Regulation of Gene Expression in Mammalian Lens Development

Transparency of the vertebrate eye lens is at least in part conferred by the short-range ordering of water-soluble crystallin molecules in the lens fiber cells. To understand the role these proteins play in maintaining lens transparency, Dr. Tsui's laboratory has been interested in identifying the mutation responsible for a dominant lens defect in the Elo (eye lens obsolescence) mouse strain. Through genetic linkage and subsequent DNA sequence analysis, a frame-shift mutation has been identified in the γE gene, the last member of the six-membered gene cluster. This observation strongly argues that γ-crystallin plays a major rather than a generally assumed passive role in lens development. Experiments are currently under way to study the dominant effect of the Elo mutation and the effect of other γ-crystallin mutations in transgenic mice.

Free-Chromatin Mapping

Based on the use of specific cell-cycle blockers and an alkaline lysis buffer, a novel procedure has been established whereby chromatin fibers could be released from interphase nuclei for physical mapping of the mammalian genome by fluorescent *in situ* hybridization. The hybridization signals from two DNA segments separated by <20 kb could be easily distinguished. This free-chromatin mapping technique has been applied in positional cloning strategies to determine the order and orientation of flanking markers and in fine structural analysis of complex sequence arrangement in the centromeric regions.

Physical Characterization of Human Chromosome 7

To generate a more complete set of reagents for the study of genes on human chromosome 7, a chromosome-specific yeast artificial chromosome (YAC) library has been constructed. Based on a human-hamster somatic cell hybrid with a single human chromosome 7, more than 1,200 YAC clones containing human DNA inserts with an average size of 500 kb have been isolated. The clones are being mapped to specific chromosome regions by hybridization with previously localized DNA segments and with a somatic cell hybrid mapping panel. More than 120 YAC clones and 15 overlapping contigs of

an average of 800 kb each have thus far been identified for a 27-Mb region (q22-q31) where specific translocation and deletion breakpoints are frequently found in leukemic cells. In addition to generation of a long-range physical map of chromosome 7, efficient techniques are being developed for detecting gene sequences based on YAC cloning.

Dr. Tsui is Senior Scientist and Sellers Chair of Cystic Fibrosis Research in the Department of Genetics at the Research Institute of the Hospital for Sick Children, Toronto, and Professor of Molecular and Medical Genetics at the University of Toronto.

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HHMI-NIH RESEARCH SCHOLARS AT THE NATIONAL INSTITUTES OF HEALTH

Since 1985 the Howard Hughes Medical Institute–National Institutes of Health Research Scholars Program has provided 271 U.S. medical students with the opportunity to experience a year of biomedical research at NIH under the tutelage of senior NIH investigators.

The program offers students a variety of unique advantages, including the ability to select a preceptor and research project after arrival at NIH; choice of a preceptor from 1,100 tenured intramural scientists in 287 laboratories working on over 2,500 research projects; educational enrichment at NIH through attendance at lectures, seminars, and presentations by renowned scientists; and collegiality with their peers while living at the Cloister, a residential and teaching facility on the NIH campus.

Applications to the 1992–1993 program increased 45% over the prior year; a total of 133 applications were received from 66 medical schools. From these applicants, a panel of senior NIH and HHMI scientists selected 72 students to be interviewed in Bethesda, Maryland. Forty-one students were accepted to the program, representing 21 medical schools. Five schools were represented for the first time: Bowman Gray School of Medicine of Wake Forest University, Brown University, the University of Hawaii, Saint Louis University, and the University of California at Irvine. In addition, nine students from the 1991–1992 class were approved to stay for a second year of scientific research. The current class of 50 scholars includes 18 women and 6 underrepresented minority students.

Class	New Students	Second-Year Students	Total
1985–1986	23	0	23
1986–1987	35	2	37
1987–1988	34	4	38
1988–1989	36	12	48
1989–1990	34	11	45
1990–1991	33	13	46
1991–1992	35	10	45
1992–1993	41	9	50

HHMI-NIH RESEARCH SCHOLARS 1992–1993

Scholar	School	Advisor	Program	Preceptor
*Scott Adler	University of Maryland	Hynda Klcinman, Ph.D.	Cell Biology	Paul E. Klotman, M.D.
John Amory	University of California, San Francisco	David Margulies, M.D., Ph.D.	Immunology	Herbert Morse, M.D.
*Susan Bardwell	Rush Medical College	Dinah Singer, Ph.D.	Immunology	Robert Hohman, Ph.D.
Maxwell Boakye	Cornell University	Monique Dubois-Dalcq, M.D.	Neuroscience	Daniel Alkon, M.D.
Alphonso Brown, Jr.	Harvard Medical School	David Margulies, M.D., Ph.D.	Immunology	Griffin Rodgers, M.D.
Edward Chan	Harvard Medical School	Stuart Rudikoff, Ph.D.	Immunology	Jonathan Vogel, M.D.
*Jason Chiu	University of Virginia	Michael Kuehl, M.D.	Genetics	Kenneth Cowan, M.D., Ph.D.
*Jeffrey Critchfield	University of California, San Francisco	Thomas Kindt, Ph.D.	Immunology	Michael Lenardo, M.D.
Eileen Deignan	Yale University	Stuart Rudikoff, Ph.D.	Ccll Biology	Juan Bonaficino, M.D.
Jordan Fieldman	Harvard Medical School	Thomas Reese, M.D.	Neuroscience	Leonardo Cohen, M.D.
Angela Fowler	Harvard Medical School	Monique Dubois-Dalcq, M.D.	Neuroscience	Marcio Chedid, Ph.D.
Katherine Frederick	University of California, Irvine	Pcter Howley, M.D.	Genetics	Andrew Geiser, Ph.D.
Yolanda Garces	University of Illinois	Robert Burke, M.D.	Neuroscicnce	Christian Felder, Ph.D.
Bang Hoang	University of California, Los Angeles	Hynda Kleinman, Ph.D.	Cell Biology	Frank Luyten, M.D.
Cara Hoffman	University of Alabama	Dale McFarlin, M.D.	Neuroscience	Susan E. Swedo, M.D.
Erich Horn	Harvard Medical School	Dinah Singer, Ph.D.	Cell Biology	Margaret Tuckcr, M.D.
*Damon Hou	University of Arizona	Hynda Klcinman, Ph.D.	Cell Biology	Allan Weissman, M.D.
Fred Hsieh	Brown University	William Eaton, M.D., Ph.D.	Ccll Biology	Michael Gottesman, M.D.
Michael Jakobsen	University of California, San Francisco	Dinah Singer, Ph.D.	Immunology	Richard B. Alexander, M.D.

Malcolm John	Harvard Medical School	Hynda Kleinman, Ph.D.	Cell Biology	Eric Verdin, M.D.
Branko Kesler	University of Pennsylvania	Dale McFarlin, M.D.	Neuroscience	Robert Balaban, Ph.D.
Paul Kim	Harvard Medical School	Thomas Reese, M.D.	Neuroscience	W. Scott Young, M.D., Ph.D.
Nerissa Ko	University of Hawaii	Robert Burke, M.D.	Neuroscience	Lynn Hudson, Ph.D.
Gregory Licholai	Yale University	Dale McFarlin, M.D.	Neuroscience	Richard Youle, Ph.D.
Cal Lin	University of California, Los Angeles	Michael Kuehl, M.D.	Immunology	Andrew Shenker, M.D., Ph.D.
Arlene Lobo	State University of New York at Buffalo	Dinah Singer, Ph.D.	Immunology	Ellis Unger, M.D.
Kreton Mavromatis	University of Michigan	Dinah Singer, Ph.D.	Immunology	Karl Munger, Ph.D.
B. Jill McFarland	Duke University	William Eaton, M.D., Ph.D.	Cell Biology	Nicholas Restifo, M.D.
*Stacey Mollis	Medical College of Ohio	Thomas Kindt, Ph.D.	Immunology	Thomas Nutman, M.D.
David Morales	Harvard Medical School	Monique Dubois-Dalcq, M.D.	Cell Biology	Hynda Kleinman, Ph.D.
Timothy Peters	Yale University	Nancy Nossal, Ph.D.	Genetics	Arthur Neinhuis, M.D.
Katherine Poehling	Bowman Gray School of Medicine of Wake Forest University	Susan Gottesman, Ph.D.	Genetics	Marc Reitman, M.D., Ph.D.
David Richardson	Harvard Medical School	Thomas Reese, M.D.	Neuroscience	Jordan Grafman, Ph.D.
Melanie Ryan	Harvard Medical School	Stuart Rudikoff, Ph.D.	Cell Biology	Keith Peden, Ph.D.
*Nicky Shah	University of Pennsylvania	Susan Gottesman, Ph.D.	Cell Biology	Richard Klausner, M.D.
Richard Shin	Northwestern University	Hynda Kleinman, Ph.D.	Cell Biology	John Brady, Ph.D.
Costi Sifri	Oregon Health Sciences University	William Eaton, M.D., Ph.D.	Cell Biology	Thomas Wellems, M.D., Ph.D.
Bernadette Smith	Ohio State University	Hynda Kleinman, Ph.D.	Cell Biology	H. Cliff Lane, M.D.
Scott Solomon	University of Maryland	Dale McFarlin, M.D.	Neuroscience	Esther Sternberg, M.D.
Jennie Sung	Northwestern University	Monique Dubois-Dalcq, M.D.	Cell Biology	Gerald Chader, Ph.D.
Stephanie Teal	University of California, San Francisco	Stuart Rudikoff, Ph.D.	Cell Biology	Richard Klausner, M.D.
Linda Tsai	Northwestern University	Michael Kuehl, M.D.	Genetics	Gerald Chader, Ph.D.
Raghuveerender Upender	University of Connecticut	Robert Burke, M.D.	Neuroscience	Barry Richmond, M.D.
Byron Van Dyke	University of California, San Francisco	Robert Nussenblatt, M.D.	Immunology	Mark Udey, M.D.
Matthew Walter	Saint Louis University	Robert Burke, M.D.	Neuroscience	Steven Jacobsen, Ph.D.
Amy Warren	University of Michigan	Michael Kuehl, M.D.	Cell Biology	Patricia Steeg, Ph.D.
Stephen Warren	University of California, Los Angeles	Robert Nussenblatt, M.D.	Immunology	Steven Rosenberg, M.D.
L. Keoki Williams	University of Michigan	Susan Gottesman, Ph.D.	Genetics	Michael Blaese, M.D.
*Michael Yanuck	Baylor College of Medicine	David Margulies, M.D., Ph.D.	Immunology	Jay Berzofsky, M.D., Ph.D.
*Dina Zand	Northwestern University	Hynda Kleinman, Ph.D.	Cell Biology	Keiko Ozato, Ph.D.

*Second year

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FORMER HHMI-NIH RESEARCH SCHOLARS

Scholar	*Medical School*	*Program Year*
Peter Abcarian	Dartmouth Medical School	1985–1986
Nicholas Abidi	University of Pennsylvania	1989–1990
David Agus	University of Pennsylvania	1990–1992
Lisa Airan	Northwestern University	1989–1991
Rhoda Alani	University of Michigan	1988–1989
Ravi Allada	University of Michigan	1990–1992
Kirsi Allison	Dartmouth Medical School	1988–1990
Christopher Ames	Georgetown University	1991–1992
Matthew Anderson	University of Miami	1987–1989
Katrin Andreasson	Columbia University College of Physician and Surgeons	1986–1987
Eddy Anglade	Yale University	1986–1987
Ann Arthur	Yale University	1989–1990
Anthony Asher	Wayne State University	1985–1986
David Bacchus	University of Southern California	1991–1992
Jeffrey Balke	University of Minnesota	1987–1988
Doris Balsamo-Hoffman	University of Oklahoma	1990–1992
Sarah Barksdale	University of California, San Francisco	1988–1989
Laurie Beitz	University of North Carolina	1987–1988
Douglas Bell	Harvard Medical School	1988–1989
Philippe Bishop	University of Nevada	1990–1991
Kevin Black	Duke University	1988–1989
Paul Bohjanen	University of Michigan	1987–1989
Stephen Boorstein	University of Michigan	1988–1990
Randy Bouligny	Cornell University Medical College	1990–1991
Andrew Boyd	University of New Mexico	1985–1986
Jodell Boyle	Duke University	1985–1986
David Bradley	University of Minnesota	1987–1989
Donna Brezinski-Caliguri	Yale University	1988–1989
James Butrynski	Wayne State University	1989–1991
John Carl III	Marshall University	1990–1991
Marc Carruth	Duke University	1991–1992
Alex Cech	Duke University	1987–1988
Paulette Chandler	Duke University	1991–1992
Catherine Chen	Harvard Medical School	1987–1988
David Chin	University of California, San Francisco	1990–1991
L. Thomas Chin	University of Massachusetts	1989–1991
Christopher Chow	Johns Hopkins University	1989–1990
Douglas Clark	University of Illinois, Chicago	1985–1987
Melissa Coale	East Carolina University	1991–1992
Jodi Cohen	Washington University	1989–1990
Joseph Cohen	Albany Medical College of Union University	1986–1987
Kenneth Colina	Baylor College of Medicine	1987–1988
Bruce Conklin	Case Western Reserve University	1986–1988
David Coran	University of Michigan	1986–1987
Christopher Corsico	Pennsylvania State University	1986–1988
Randall Cron	University of California, Los Angeles	1986–1988
Anita Dash	Northeastern Ohio Universities College of Medicine	1989–1990
Lourdes de Armas	University of Puerto Rico	1988–1989
D. Scott Donnelly	Duke University	1991–1992
Susan Dorman	Duke University	1991–1992
Brock Eide	University of Washington	1985–1986
Richard Ellis	Southern Illinois University	1988–1989
John Eng	University of Wisconsin	1987–1989
Emad Eskandar	University of Southern California	1989–1991
Alik Farber	Harvard Medical School	1990–1991
Elizabeth Farr	University of Michigan	1988–1990
Douglas Feltner	University of Michigan	1986–1987
Michael Ferrick	University of Michigan	1989–1990

Gregory Fox	University of Michigan	1985–1986
Ahron Friedberg	State University of New York at Buffalo	1987–1988
David Frucht	Duke University	1988–1990
Alicia Fry	University of Cincinnati	1987–1989
Sarah-Jo Gahm	University of Vermont	1989–1991
Donald Gilbert	University of Michigan	1991–1992
John Gilstad	Uniformed Services University of the Health Sciences	1989–1991
Adam Golden	University of Miami	1990–1991
Gary Gottesman	University of Michigan	1985–1986
Kenneth Graham	University of Alabama	1990–1992
William Greene	University of California, San Francisco	1988–1989
David Gudeman	University of Kansas	1986–1987
Heidi Hagman	Oregon Health Sciences University	1989–1990
William Harbour	Johns Hopkins University	1987–1988
Raymond Haroun	Columbia University	1991–1992
Laura Harris	East Carolina University	1990–1991
Adam Hartman	Northwestern University	1991–1992
Hirofumi Hashimoto	Stanford University	1989–1991
Hratchia Havoundjian	University of North Carolina	1985–1986
Donald Heck	Duke University	1990–1992
John Hegarty	Pennsylvania State University	1988–1989
Garrett Hisatake	University of Utah	1991–1992
Victor Ho	Duke University	1985–1986
Norman Hogikyan	University of Michigan	1985–1986
Helen Hollingsworth	New York University	1986–1987
Mark Holm	University of Minnesota	1987–1988
John Houston	Yale University	1991–1992
Eric Hsi	University of Michigan	1987–1988
Paul Hsieh	University of Michigan	1986–1987
James Huang	Baylor College of Medicine	1987–1988
Carol Ann Huff	Baylor College of Medicine	1988–1990
Burt Hutto	Medical University of South Carolina	1986–1987
Laura Ibsen	University of California, San Diego	1986–1987
Carlos Ince	Duke University	1990–1991
Suzanne Jan de Beur	George Washington University	1989–1990
Eric Janis	Johns Hopkins University	1987–1988
Michael Johnson	University of Virginia	1989–1991
Aarchan Joshi	Robert Wood Johnson	1991–1992
Eric Kaldjian	University of Michigan	1986–1987
Sang-Mo Kang	Harvard Medical School	1989–1991
Robert Kanterman	University of Miami	1988–1990
Stamatina Kaptain	Harvard Medical School	1989–1991
Velissarios Karacostas	Eastern Virginia Medical School	1987–1989
Judy Kim	Johns Hopkins University	1987–1988
James Kirby	University of Pennsylvania	1989–1991
David Koeplin	University of Michigan	1989–1990
Vera Kowal	University of Michigan	1986–1987
Alan Krasner	Northwestern University	1985–1986
Jody Krosnick	Temple University	1987–1988
Richard Kuno	University of California, San Francisco	1990–1991
Sandeep Kunwar	University of California, San Francisco	1991–1992
Scott Lankford	University of Maryland	1990–1991
Robert Lechleider	University of Illinois, Chicago	1988–1989
Brian Lee	University of California, Los Angeles	1991–1992
Christine Lee	Duke University	1986–1987
Joon Lee	Duke University	1986–1987
Mitchell Douglas Lee	East Carolina University	1988–1989
John Lepore	Harvard Medical School	1990–1991
Caroline Leppin-Davison	George Washington University	1990–1991
Janet Lewis	Medical College of Ohio	1985–1986
Andrew Li	George Washington University	1989–1990
Thomas Lietman	Columbia University College of Physicians and Surgeons	1987–1989
Ajit Limaye	University of Washington	1988–1990
Bertha Lin	University of Michigan	1988–1989

Paul Lin	University of Mississippi	1989–1990
William Lipham	Baylor College of Medicine	1988–1990
Matthew Lonergan	Harvard Medical School	1990–1992
Susan Lontkowski	Duke University	1986–1987
Richard Lopez	Stanford University	1985–1987
Alice Ma	University of Michigan	1986–1987
Steven Maxfield	Duke University	1987–1988
David McDermott	University of Virginia	1989–1990
Josh McDonald	Duke University	1988–1989
Patrick McQuillen	University of California, San Diego	1990–1991
Michael Measom	University of Utah	1986–1987
Manuel Mendoza	University of Wisconsin	1990–1991
Mark Miller	Yale University	1988–1989
Matthew Mitchell	Harvard Medical School	1989–1990
Michael Moates	University of Alabama	1987–1988
D. Branch Moody	Johns Hopkins University	1991–1992
Lewis Morgenstern	University of Michigan	1987–1988
Bobak Mozayeni	Albany Medical College of Union University	1985–1986
Michael Myers	University of Michigan	1985–1986
Elias Najem	University of Medicine and Dentistry of New Jersey	1986–1987
Laura Napolitano	University of Rochester	1987–1989
Kathleen Newell	University of Kansas	1988–1990
Susan Nicholson	University of Pittsburgh	1989–1990
Bert O'Neil	University of California, Los Angeles	1991–1992
Angelica Oviedo	Northwestern University	1990–1992
Leslie Parent	Duke University	1985–1986
Carmen Parrott	University of California, San Francisco	1988–1989
Maitray Patel	University of Michigan	1985–1986
Shaila Patel	Ohio State University	1989–1990
Christian Pavlovich	University of California, San Francisco	1991–1992
David Pezen	Loyola-Stritch School of Medicine	1987–1989
Andrew Phillips	Yale University	1989–1990
Caroline Philpott	Duke University	1985–1986
Roberto Pineda II	University of Minnesota	1989–1990
David Polomis	University of Michigan	1986–1987
David Pombo	University of Utah	1986–1987
Daniel Press	University of Connecticut	1991–1992
Christopher Rall	Medical College of Virginia	1986–1987
Sridhar Ramaswamy	Boston University	1990–1991
Peter Redford	University of Kansas	1990–1991
André Reed	University of Washington	1990–1992
Michael Rees	University of Michigan	1987–1989
Leslie Reynolds	Duke University	1989–1990
Jesus Rivera-Nieves	University of Puerto Rico	1988–1989
Mark Robien	Tufts University	1990–1992
Edwin Rock	University of Pittsburgh	1985–1986
Mitchell Rosner	Harvard Medical School	1988–1990
Paula Ross	University of Michigan	1986–1987
Tanya Rutledge	Harvard Medical School	1989–1991
Mark Sands	Northwestern University	1987–1988
George Sarosi	Harvard Medical School	1990–1991
Steven Schafer	University of Michigan	1990–1991
Andrew Scharenberg	University of Michigan	1987–1988
Michael Schneck	Cornell University	1985–1986
Lisa Schroepfer	University of Florida	1991–1992
Todd Seidner	University of California, Los Angeles	1989–1990
Norman Sharpless	University of North Carolina at Chapel Hill	1990–1991
Belinda Shirkey	University of Arkansas	1991–1992
Scott Simpson	University of Texas, Galveston	1988–1989
Photini Sinnis	Dartmouth Medical School	1986–1987
Stephen Skirboll	University of Pennsylvania	1987–1988
Melisande Smith	Dartmouth Medical School	1990–1991
Spencer Smith	Duke University	1987–1989
Gregory Springett	Harvard Medical School	1987–1988
John Stahl	Duke University	1989–1990

Virginia Stark-Vancs	University of Oklahoma	1985–1986
Douglas Steinbrech	University of Iowa	1991–1992
Jeffrey Sussman	University of California, Los Angeles	1986–1987
David Tanen	New York University	1988–1989
Marie Tani	Case Western Reserve University	1986–1987
Darryl Tannenbaum	Harvard Medical School	1990–1991
James Teener	University of Michigan	1987–1988
Charles Tsai	University of Michigan	1986–1987
Prashanth Vallabhanath	University of Michigan	1991–1992
Michelle Verplanck	Michigan State University	1987–1989
Mary Jo Viglione	University of Pennsylvania	1985–1986
Joseph Vinetz	University of California, San Diego	1988–1989
John Viola	Jefferson Medical School	1987–1988
Darshan Vyas	University of Michigan	1990–1991
Kathleen Waite	Duke University	1988–1989
Mark Walker	Johns Hopkins University	1990–1992
Philip Wang	Harvard Medical School	1986–1987
Jill Watanabe	Johns Hopkins University	1988–1989
Louis Weimer	Emory University	1986–1987
David Weiner	State University of New York at Buffalo	1988–1990
Rosalind Welty	University of Michigan	1986–1987
Keith Wharton	University of Arizona	1988–1989
Holly Williams	University of California, San Francisco	1990–1991
Steven Wolff	Duke University	1986–1988
Jane Wong	Tulane University	1988–1989
Rex Wong	University of Michigan	1989–1990
Michael Wood	University of Michigan	1986–1987
Erica Wu	University of Michigan	1991–1992
Samuel Wu	Harvard Medical School	1989–1991
Victor Wu	Johns Hopkins University	1988–1989
Virginia Yip	University of Illinois	1991–1992
Lourdes Ylagan	Ohio State University	1991–1992
Stephanie Telesetsky Young	Duke University	1988–1989
Suzanne Zorn	Pennsylvania State University	1986–1987

In 1987 the Institute established the Office of Grants and Special Programs to help strengthen education in biology and the related sciences, complementing its medical research activities. A major objective of the grants program is to recruit and retain students in biomedical research and education and thereby foster scientific discovery.

The Institute supports grant programs in graduate, undergraduate, and precollege science education and research in the United States and abroad. In addition, the Office is undertaking a comprehensive assessment program.

GRADUATE EDUCATION IN BIOLOGICAL SCIENCES

Graduate education activities are aimed at expanding the pool of outstanding biomedical investigators, including physician-scientists, through the awarding of individual fellowships. In addition, a small number of grants have been awarded to selected institutions that have traditionally played a unique international role in biological science education and research training. After five years of grants program awards, the fellowship programs are now operating at steady-state levels. Over $11.5 million went to the support of about 500 fellows in the Institute's three graduate science education fellowship programs during the 1992 fiscal year.

Predoctoral Fellowships in Biological Sciences

The goal of the Predoctoral Fellowships in Biological Sciences Program is to promote excellence in biomedical research by helping prospective researchers with exceptional promise obtain high-quality graduate education. Fellowships are awarded for full-time study toward a Ph.D. or Sc.D. degree in the biological sciences that parallel the Institute's scientific program areas. Each predoctoral fellowship provides an annual stipend for up to five years and an annual cost-of-education allowance to the institution at which the fellow will study. The fellowships are intended for students at the beginning of their graduate study. Those eligible at the time of application may be college seniors, college graduates with no or limited postbaccalaureate study in the biological sciences, or first-year graduate students, including foreign citizens as well as U.S. citizens. Students who hold or are pursuing degrees in medicine, veterinary medicine, or dentistry also may apply for fellowships for study toward the Ph.D. degree.

In the 1992 fellowship competition, about 1,400 eligible applicants were evaluated by panels of distinguished biomedical scientists convened by the National Research Council of the National Academy of Sciences, which administers the competition on behalf of the Institute. Based on these evaluations, the Institute selected 70 fellows.

Drawn from 47 undergraduate institutions (including 6 outside the United States), the new fellows are undertaking graduate study at 23 academic institutions throughout the country and abroad. This past year, 297 predoctoral fellows and the 52 universities they are attending received over $7 million for support of the fellows' graduate studies. The Institute will award about 66 new fellowships annually, so that approximately 330 predoctoral fellows will be supported in any given year.

New Awards—Predoctoral Fellow

Biochemistry and Structural Biology
   David Min Chao, *Massachusetts Institute of Technology*
   Melinda Bonnie Fagan, *Stanford University*
   David Henry Hackos, *University of California, San Francisco*
   Stuart Spencer Licht, *Massachusetts Institute of Technology*
   I-Fan Theodore Mau, *University of California, San Francisco*
   Kayvan Roayaie, *University of California, San Francisco*
   Jennifer Ellen Schmidt, *University of Washington*
   Kyle John Vogan, *McGill University, Canada*
   Edgar Chong Young, *Brandeis University*
   Karen Marie Zito, *University of California, Berkeley*

Biostatistics, Epidemiology, and Mathematical Biology
   Anne M. Bronikowski, *The University of Chicago*
   Michael Joseph Daniels, *Harvard University*

Cell Biology and Immunology
   Jose Antonio Alcantara, *University of Pennsylvania*
   Lara Jane Ausubel, *Harvard University*
   Michelle Leigh Boytim, *Stanford University*
   Bradley Brian Brasher, *Harvard University*
   George Yen-Hsi Liu, *University of Cambridge, England*
   Ellen Annette Lumpkin, *University of Texas Southwestern Medical Center at Dallas*

Thomas Nicholas Moreno, *University of California, San Diego*
Helen Chun-Hui Su, *Brown University*
Jennifer Jo Wernegreen, *Yale University*
Carol Norris White, *University of Connecticut*

Genetics, Microbiology, Molecular Biology, and Virology
Adam Henry Amsterdam, *Massachusetts Institute of Technology*
Kristin Kay Baldwin, *Stanford University*
Hsiuchen Chen, *Harvard University*
John Shichieh Chuang, *University of California, San Francisco*
Francesca Cole, *Massachusetts Institute of Technology*
Gene Cutler, *University of California, Berkeley*
Judith Kimberly Davie, *University of California, Berkeley*
Arshad Bachubhai Desai, *University of California, San Francisco*
Douglas David Fenger, *Massachusetts Institute of Technology*
Michael Jonathan Ford, *Cornell University*
Kenneth Adam Frauwirth, *University of California, Berkeley*
Laura Susan Gammill, *Massachusetts Institute of Technology*
Tracy Lanise Johnson, *University of California, Berkeley*
Olivia Guadalupe Kelly, *Harvard University*
Corwin Francis Kostrub, *Harvard University*
Mark Mordecai Metzstein, *Massachusetts Institute of Technology*
Anh Tuan Nguyen-Huynh, *Harvard University*
Allison Joy Oppenheimer, *Harvard University*
Robin Rae Pals, *Northwestern University*
Adam Daniel Rudner, *University of California, San Francisco*
Linda April Stillman, *The University of Chicago*
Christian J. Ullsperger, *University of California, Berkeley*
Leticia Rosa Vega, *Massachusetts Institute of Technology*
Sonya Marie Vieira, *The Johns Hopkins University*
Diana Elaine Wofford, *University of California, San Diego*

Neuroscience, Biophysics, Developmental Biology, Pharmacology, and Physiology
Michael Stephen Beauchamp, *University of California, San Diego*
Mark Christopher Bieda, *Stanford University*

Solange Pezon Brown, *Harvard University*
Jennifer Arwen Cummings, *University of California, San Francisco*
Hannah Alice Dvorak, *California Institute of Technology*
Eva Marie Finney, *University of California, Berkeley*
Joanne Fraher, *University of Pennsylvania*
Yuval Gazit, *Massachusetts Institute of Technology*
Takao Kurt Hensch, *University of California, San Francisco*
Anton Edward Krukowski, *University of California, San Francisco*
Matthew Swan Lawrence, *Stanford University*
Jennifer Fran Linden, *California Institute of Technology*
Henry Wiltshire Mahncke, *University of California, San Francisco*
Jennifer Ann Martino, *Rutgers, the State University of New Jersey, New Brunswick*
Mireya Nadal-Vicens, *Stanford University*
Rhett Valino Pascual, *University of California, Berkeley*
Deborah Melissa Redish, *Massachusetts Institute of Technology*
Naomi Leah Ruff, *University of California, San Diego*
Chris Schafmeister, *University of California, San Francisco*
Christine Theresia Schulteis, *University of California, Los Angeles*
Martin Bernard Stemmler, *California Institute of Technology*
Ramon Kenneth Tabtiang, *University of California, San Francisco*
Jennifer Wahlsten, *University of Minnesota–Twin Cities*

Continuing Awards—Predoctoral Fellows
Biochemistry and Structural Biology
Mark William Albers, *Harvard University*
George Cyrus Allen, Jr., *Stanford University*
Stephen Francis Anderson, *Yale University*
Todd Sterling Anderson, *Massachusetts Institute of Technology*
Amir Attaran, *University of Oxford, England*
Douglas Edward Barrick, *Stanford University*
Gregory Joseph Beitel, *Massachusetts Institute of Technology*
Kirsten Beatrice Bibbins, *University of California, San Francisco*
Rodney Dean Boyum, *Harvard University*

James William Bryson, *Northwestern University*

Michael Joseph Campbell, *Stanford University*

Vivien Wai-Fan Chan, *University of California, San Francisco*

Mark Louis Dell'Acqua, *Harvard University*

Abby Felicia Dernberg, *University of California, San Francisco*

Leonard Roger Duncan, *Harvard University*

Tanya Grace Falbel, *University of Colorado at Boulder*

Eric Benjamin Fauman, *University of California, San Francisco*

Antoine Alfred Firmenich, *Stanford University*

Ernest Samuel Fraenkel, *Massachusetts Institute of Technology*

Shachar Frank, *University of Illinois at Urbana-Champaign*

Kevin Hastings Gardner, *Yale University*

Levi Alexander Garraway, *Harvard University*

Razmic Sarkies Gregorian, Jr., *Yale University*

Stacey Lynn Harmer, *University of California, San Francisco*

Norah Lynn Henry, *Stanford University*

Lorraine Dee Hernandez, *University of California, San Francisco*

David Alan Hinds, *Stanford University*

Alec Eaton Hodel, *Yale University*

Andrew Henry Huber, *California Institute of Technology*

Thomas Joseph Kappock IV, *Yale University*

Mark Andrew Lemmon, *Yale University*

Wallace Frank Marshall, *University of California, San Francisco*

Andrew G. Morton, *University of Oregon*

Seong-Joon Park, *Harvard University*

Pieter Maurits Pil, *Massachusetts Institute of Technology*

Leonard Douglas Pysh, *University of California, San Diego*

David Israel Resnick, *Massachusetts Institute of Technology*

David Carlton Schweisguth, *Yale University*

Andrew Kwan-Nan Shiau, *University of California, San Francisco*

Natasha Mary Simkovich, *Harvard University*

Jonathan Prescott Staley, *Massachusetts Institute of Technology*

Troy Edward Wilson, *University of California, Berkeley*

Hao Wu, *Purdue University*

Samuel Sumu Wu, *Stanford University*

Patrick Parvis Zarrinkar, *Massachusetts Institute of Technology*

Biostatistics, Epidemiology, and Mathematical Biology

Christian Benjamin Bynum, *University of Washington*

Sharon Lynn Deem, *University of Florida*

Nancy Lynne Paul, *Carnegie Mellon University*

Cell Biology and Immunology

Sarki Abba Abdulkadir, *The Johns Hopkins University*

Srinivas Akkaraju, *Stanford University*

Janet Lynn Baird, *University of Pennsylvania*

Jon Anthony Buras, *Boston University*

Rebecca Dawn Burdine, *Yale University*

Natasha Grant Deane, *University of Texas Medical Branch at Galveston*

David Benjamin Fruman, *Harvard University*

Carlos Joaquin Gimeno, *Massachusetts Institute of Technology*

Lisa Glickstein, *Cornell University Medical Center*

Jodi Elisabeth Goldberg, *Stanford University*

William Chun Hahn, *Harvard University*

Lora Virginia Hooper, *Washington University*

Mark Edward Johnson, *Rush University*

William James Joiner, *Yale University*

Eugene Hideo Kaji, *Massachusetts Institute of Technology*

Jaana Terhikki Karttunen, *University of California, Berkeley*

Leslie Susan Kean, *Emory University*

Hubert Tackyoung Kim, *Stanford University*

Julie Ann Lang, *University of Colorado Health Sciences Center*

Johnson E-Zye Loh, *Yale University*

John Adrian Lunn, *Yale University*

Kevin Thomas Madden, *Yale University*

Jennifer Michaelson, *Albert Einstein College of Medicine of Yeshiva University*

Sean Joseph Morrison, *Stanford University*

Benjamin David Ortiz, *Stanford University*

Jonathan Gaertner Pope, *Northwestern University*

Andrew Mark Posselt, *University of Pennsylvania*

Kris Alan Reedquist, *Harvard University*

Edwin Palmer Rock, *Stanford University*

Walter Kenneth Schmidt, Jr., *University of California, Berkeley*

Miriam Cather Schneebeck, *University of New Mexico, Albuquerque*

Melanie Ann Sherman, *Emory University*

Jill Caron Sible, *Tufts University School of Medicine*

Steven Mitchell Singer, *Stanford University*
Amy Lea Teel, *University of Minnesota–Twin Cities*
Lisa Wessendorf, *University of Cambridge, England*
Jacob Nathaniel Wohlstadter, *Harvard University*
Laurence Mitchell Zeitlin, *The Johns Hopkins University*
Wei Zhang, *Harvard University*

Genetics and Molecular Biology
Kamran Ahmad, *University of Utah*
Dianne M. Ahmann, *Massachusetts Institute of Technology*
Hiroshi Akashi, *The University of Chicago*
Curtis R. Altmann, *University of California, Berkeley*
Nicanor Robles Austriaco, Jr., *Massachusetts Institute of Technology*
Maria-Ines Benito, *Stanford University*
Robert Louis Bertekap, Jr., *University of Pennsylvania*
Seth Blackshaw, *The Johns Hopkins University*
John Patrick Bodeau, *Stanford University*
William Wayne Bogan, Jr., *University of Wisconsin–Madison*
Brian Michael Cali, *University of Wisconsin–Madison*
Brendan Patrick Cormack, *Harvard University*
Elizabeth M. Crough, *Case Western Reserve University*
Deborah Ann DeRyckere, *University of California, Berkeley*
Alexander Dornfield Diehl, *Washington University*
Monicia Ann Elrod, *Massachusetts Institute of Technology*
Douglas McIntosh Fambrough, *University of California, Berkeley*
John Edward Fowler, Jr., *University of California, Berkeley*
Katherine Louise Friedman, *University of Washington*
Ellen L. Gadbois, *Massachusetts Institute of Technology*
Joseph Gerard Hacia, *California Institute of Technology*
Karen Gwen Hales, *Stanford University*
Midori Akiko Harris, *Cornell University*
Junjiro Horiuchi, *Massachusetts Institute of Technology*
Weidong Huang, *Harvard University*
Gene Simon Huh, *Massachusetts Institute of Technology*

Laurie Issel-Tarver, *University of California, Berkeley*
Shera Felice Kash-Anderlik, *Baylor College of Medicine*
Patrick John Krysan, *Stanford University*
Laura Faye Landweber, *Harvard University*
Mark Lee, *Harvard University*
Stephen Ek Teong Loo, *University of California, Berkeley*
Julin Nassir Maloof, *University of California, San Francisco*
Gerard Michel Manning, *Stanford University*
Paul Calburt Marker, *Stanford University*
Anne Elizabeth McBride, *University of Colorado at Boulder*
David Bernard Miklos, *Yale University*
Antonio Alexander Monterrosa, *University of Colorado at Boulder*
Nancy Faye Strom Morgan, *Yale University*
Aaron Michael Neiman, *University of California, San Francisco*
Stephen Christopher Ogg, *University of California, San Francisco*
Jennifer Anne Ostrom, *Washington University*
Scott Russell Panzer, *University of California, Berkeley*
Lisa Marie Parker, *University of California, Berkeley*
Janice Diane Pata, *University of Colorado at Boulder*
Pratima Lalita Raghunathan, *University of California, San Francisco*
Ilaria Leslie Rebay, *Yale University*
David John Riese, *Yale University*
Mark Paul Running, *California Institute of Technology*
Manuel Benito Sainz, *University of Oregon*
Sofie Reda Salama, *University of California, Berkeley*
David Ben Schauer, *Stanford University*
Kathleen Winnifred Schleifer, *University of Wisconsin–Madison*
Anthony Schwacha, *Harvard University*
Tricia Renee Serio, *Yale University*
Susan Shannon, *University of Oregon*
Lisa Becker Smit, *University of Michigan–Ann Arbor*
Tracy Lynn Smith, *Massachusetts Institute of Technology*
Jonathan Micah Solomon, *Massachusetts Institute of Technology*
Eric Vincent Stabb, *University of Wisconsin–Madison*
Gillian Marie Stanfield, *Massachusetts Institute of Technology*

Ben Stanger, *Harvard University*

Julie Anne Theriot, *University of California, San Francisco*

Jeffrey Denis Thomas, Jr., *Massachusetts Institute of Technology*

Willem Jan van Heeckeren, *Harvard University*

Daniel Choe Weinstein, *Rockefeller University*

Rush Spencer Wells IV, *Harvard University*

Michael Andreas Welte, *The University of Chicago*

Ann-Marie White, *Massachusetts Institute of Technology*

Wayne Wei-En Wu, *University of California, Berkeley*

Tammie C. Yeh, *Stanford University*

Man Lun Yip, *California Institute of Technology*

Ilan David Zipkin, *University of California, San Francisco*

Neuroscience, Developmental Biology, and Physiology

Ralph Adolphs, *California Institute of Technology*

Matthew Linley Anderson, *Yale University*

Joel Bard, *Harvard University*

James William Booth, *Harvard University*

Kenneth Ashton Callicott, *Stanford University*

Robert Keith Cerpa, *University of California, San Francisco*

John-Marc Chandonia, *Harvard University*

Michael Allen Chastain, *University of California, Berkeley*

Eduardo Jose Chichilnisky, *Stanford University*

Marina Esther Chicurel, *Harvard University*

Gal Abraham Cohen, *Stanford University*

Sophia Alison Colamarino, *University of California, San Francisco*

Yang Dan, *Columbia University*

Alan Dardik, *University of Pennsylvania*

Rudi Edward De Koker, *Stanford University*

Erik-Robert Evensen, *Harvard University*

Sarah McEwen Farrington, *Harvard University*

Daniel Eric Feldman, *Stanford University*

Robert Charles Gensure, *Tulane University*

Robert Walter Gereau, *Emory University*

Miriam Beth Goodman, *The University of Chicago*

Carl Lee Gordon, *Massachusetts Institute of Technology*

Harvey Allan Greisman, *Massachusetts Institute of Technology*

Karen Florence Han, *University of California, San Francisco*

Laura Kelly Vinson Harper, *The Johns Hopkins University*

Beth Brianna Hogans, *University of Maryland at Baltimore*

Charles Garrett Hoogstraten, *University of Wisconsin–Madison*

Edward Wei-Chieh Hsu, *The Johns Hopkins University*

Robin Anne Huff, *Duke University*

Jorge Alberto Iniguez-Lluhi, *University of Texas Southwestern Medical Center at Dallas*

Anne Katherine Knecht, *University of California, Berkeley*

Zoran Kurtovic, *University of California, Berkeley*

James Andrew Langeland, *University of Wisconsin–Madison*

Christopher John Lee, *Stanford University*

David Nathan Lieberman, *Stanford University*

James Eduard Mace, *University of California, San Francisco*

Zachary Frank Mainen, *University of California, San Diego*

Nicholas Reed Marsh-Armstrong, *Harvard University*

Raymond Richard Mattingly, *University of Virginia*

Audrea Kay Merchant, *University of Michigan–Ann Arbor*

Jane Elizabeth Minturn, *Yale University*

Lawrence Christopher Myers, *Harvard University*

Darin Arnold Nelson, *Duke University*

James Charles Nieh, *Cornell University*

Thomas Stephen Otis, *Stanford University*

Maria Rhea Parkhurst, *The Johns Hopkins University*

Gina Rochelle Poe, *University of California, Los Angeles*

Rene Carlos Renteria, *Yale University*

Eric Whitney Sayers, *Yale University*

T. Eric Schackow, *Northwestern University*

James Mark Schuster, *Duke University*

Stanley Yang Shaw, *Harvard University*

Jason Ben Shear, *Stanford University*

Diana Kathryn Smetters, *Massachusetts Institute of Technology*

Gerald Troy Smith, *University of Washington*

Leslie Karen Sprunger, *University of Minnesota–Twin Cities*

Davida Zara Streett, *The Johns Hopkins University*

Paul Dennis Thomas, Jr., *University of California, San Francisco*

Elaine Ellen Thompson, *The Johns Hopkins University*

Jack Wei-Tan Tsao, *University of Oxford, England*
Monica Lynn Vetter, *University of California, San Francisco*
Timothy James Walter, *University of Virginia*
Mari Watanabe, *Cornell University*
Grace Emay Wei, *The University of Chicago*
David Kreigh Welsh, *Harvard University*

Research Training Fellowships for Medical Students

To strengthen and expand the pool of medically trained researchers, the Research Training Fellowships for Medical Students Program was launched in the fall of 1988. The program enables selected applicants who have developed an interest in fundamental research during the course of their medical studies to spend an intensive research year in a laboratory. Complementing the HHMI-NIH Research Scholars Program located in Bethesda, the grants program provides opportunities for up to 60 fellows each year to engage in full-time research at any medical school, university, or not-for-profit research institute in the United States except NIH.

On the basis of review by a panel of eminent academic scientists and physicians, the Institute named 60 new medical student fellows in the 1992 competition. In addition, four of the 1991 fellows were awarded support for a second year of research. The fellows, drawn from 22 medical schools, are pursuing their research training at 18 academic institutions in the United States. The award provides a stipend to the fellow, a research allowance for the student's mentor, and an allowance to the fellowship institution.

In addition, 22 of the 1991 fellows and Research Scholars were selected for up to two years of fellowship support upon return to their medical studies. Fifteen continued fellows selected the prior year will receive a second year of such continued support. For the continued fellows, the Institute provides stipends and an education allowance to their medical schools to help cover tuition and other expenses.

Support for medical student fellows, including both initial awards and continued awards, during fiscal year 1992 totaled over $2 million.

New Awards—Medical Student Fellows
Biochemistry and Structural Biology
Andrew C. Hecht, *Harvard University*
Michael George Jacoby IV, *Washington University*
Cindy Levine, *Cornell University Medical Center*
Melinda Aileen Maggard, *Harvard University*

Biostatistics, Epidemiology, and Mathematical Biology
Kenneth Charles Goldberg, *Medical College of Wisconsin*

Cell Biology and Immunology
Peter Alan Barton, *University of Michigan–Ann Arbor*
Tamara Nathan Bloch, *The University of Chicago*
Judson Michael Brandeis, *Harvard University (Vanderbilt University)**
Brian Eugene Brigman, *University of North Carolina at Chapel Hill*
Christopher Hayden Cabell, *Duke University*
John Dwight Chen, *Stanford University*
Briggs Edward Cook, *The Johns Hopkins University*
Michael Jay Fisher, *Harvard University*
Tony Friedman, *Duke University*
Tejal Kanti Gandhi, *Harvard University*
Robert Ronald Hergan, Jr., *University of Pennsylvania*
Jennifer Lynn Hunter, *Duke University*
Rainu Kaushal, *Harvard University*
Sunjay Kaushal, *Harvard University (The Johns Hopkins University)**
Daniel Lewis Kraft, *Stanford University*
Phillip R. Kravetz, *Harvard University (University of Michigan–Ann Arbor)**
Katherine Paige Lemon, *Harvard University*
Steven P. Leon, *Harvard University*
James M. Malone III, *University of Nebraska Medical Center*
Ryland Melford III, *Washington University*
Michael E. Mitchell, *Harvard University*
John Edwin Monks, *Duke University*
Sam Peyvand Mostafapour, *Stanford University*
George Braxton Payne, *Duke University*
Amy Estelle Pickar, *Duke University*
Barbara Ann Pippin, *University of Pittsburgh*
Stacy Lynne Smith, *Harvard University*
Kimberly Crapo Stone, *Duke University*
Renee J. Strucke, *Harvard University*
William Hudson Sweatt, *The Johns Hopkins University*
Alison Patricia Toth, *Duke University*

Genetics and Molecular Biology
Hugh B. Carey, *Yale University (University of Connecticut)**
Rohan Hazra, *The Johns Hopkins University*
Catherine Marshall Hurt, *University of California, San Francisco*
Jennifer Ellen Lawrence, *The Johns Hopkins University*

550

Benhur Lee, *Yale University*

John Halvor Lee, *University of Minnesota–Twin Cities*

Kristi Levine, *Cornell University Medical Center*

Victoria Alexandra Mancuso, *University of California, San Francisco*

William Hikaru Matsui, *University of California, San Francisco*

Mitra Maybodi, *Duke University*

Margaret Elise McLaughlin, *Harvard University (University of Pennsylvania)**

Jennifer Lee Willert, *University of California, San Francisco*

Julie Anne Wissink, *Stanford University*

Neuroscience and Physiology
Nicholas M. Boulis, *Harvard University*
Mark Anthony Hester, *Duke University*
John Victor Heymach, *Stanford University*
Brian Frederick Hoeflinger, *Medical College of Ohio*
Sigmund Huang Hsu, *Brown University*
Delphine Hu, *Harvard University (Yale University)**
Patti Chia-Sue Huang, *Duke University*
Kimberly Susan Kauffman, *Duke University*
Lori Jean Kutka, *Washington University*
Christine G. Lydon, *Yale University*
Elizabeth Mwikali Mutisya, *Harvard University*

*A medical school affiliation other than the fellowship institution is indicated in parentheses.

Continued Awards—Second Year of Research
Biochemistry and Structural Biology
Agnes Marks Cartner, *University of Alabama at Birmingham*
Barbara Ann Zylbert, *Stanford University*

Genetics and Molecular Biology
Anne-Marie Brillantes, *Mount Sinai School of Medicine of the City University of New York*

Neuroscience and Physiology
Jeffrey A. Guy, *Harvard University*

Continued Awards—Medical Student Fellows, Return to Medical Studies
Lisa Elizabeth Airan, *Northwestern University*
Ravi Allada, *University of Michigan–Ann Arbor*
Sharon Ann Bloom, *Stanford University*
Marc Carruth, *Duke University*
Marc Ivan Diamond, *University of California, San Francisco*
Emad Nader Eskandar, *University of Southern California*

Evelyn Mary Gonzalez, *Harvard University*
Ronell Allen Hansen, *University of Minnesota–Twin Cities*
Raymond Issac Haroun, *Columbia University*
Laura Lynette Harris, *East Carolina University*
Hirofumi Hashimoto, *Stanford University*
Donald Vincent Heck, *Duke University*
Erica W. Hwang, *University of Michigan–Ann Arbor*
Hanlee P. Ji, *The Johns Hopkins University*
Bamidele Omowunmi Kammen, *Harvard University*
Stamatina Kaptain, *Harvard University*
James Edward Kirby, *University of Pennsylvania*
Richard Paul Kosman, *Harvard University*
Sandeep Kunwar, *University of California, San Francisco*
Edward Earl Leonard II, *University of Pittsburgh*
Vincent Wei-Tsin Li, *Harvard University*
Matthew Lonergan, *Harvard University*
Marga Faith Massey, *Duke University*
Samuel Mark Moskowitz, *Harvard University*
Lisa Michelle Owens, *University of North Carolina at Chapel Hill*
Michael Angelo Panzara, *Stanford University*
Christian Paul Pavlovich, *University of California, San Francisco*
Daniel A. Peterson, *Rush University*
Michael James Polydefkis, *The Johns Hopkins University*
Vivek Yerrapu Reddy, *University of Michigan–Ann Arbor*
Mark Andrew Robien, *Tufts University School of Medicine*
C. Daniel Salzman, *Stanford University*
Christine Marie Seroogy, *University of Minnesota–Twin Cities*
Mark Frederick Walker, *The Johns Hopkins University*
Holly Lynn Williams, *University of California, San Francisco*
David William Wright, *University of Alabama at Birmingham*
Catherine Ju-Ying Wu, *Stanford University*

Postdoctoral Research Fellowships for Physicians

As another means of strengthening the pool of medically trained researchers, a program of Postdoctoral Research Fellowships for Physicians was launched in the fall of 1989. It supports physicians seeking full-time training in fundamental research. About 25 fellowships are awarded annually. They provide three years of support, including stipends and research and institutional allowances. Physi-

cians who at the start of the fellowship will have completed at least two years of postgraduate clinical training and no more than two years of postdoctoral research training are eligible for awards, including U.S. and foreign citizens.

On the basis of review by a panel of distinguished physician-scientists and biologists, the Institute selected 30 fellows in the 1992 competition and appointed 4 associates in Howard Hughes Medical Institute laboratories. These physicians will undertake their research at 22 institutions across the United States and abroad beginning September 1992 or later. Forty-six fellows from the 1990 and 1991 competitions received over $2.6 million in support under the grants program during fiscal year 1992.

New Awards—Postdoctoral Research Fellowships for Physicians

Cell Biology and Regulation

William Bishai, M.D., Ph.D., *The Johns Hopkins University School of Medicine*

Joseph Bokar, M.D., Ph.D., *Case Western Reserve University*

George Cotsarelis, M.D., *University of Pennsylvania*

Robert D'Amato, M.D., Ph.D., *Children's Hospital, Boston*

David Frank, M.D., Ph.D., *Harvard Medical School*

Mary Gray, M.D., *Northern California Institute for Research and Education*

Antonio Iavarone, M.D., *University of California, San Francisco*

Dean Kedes, M.D., Ph.D., *University of California, San Francisco**

John Krege, M.D., *University of North Carolina at Chapel Hill*

Jose Leis, M.D., Ph.D., *Dana-Farber Cancer Institute*

Thomas McGarry, M.D., Ph.D., *University of California, San Francisco*

Genetics

Johanna Daily, M.D., *Harvard School of Public Health*

Douglas Feltner, M.D., *National Institutes of Health, National Institute of Child Health and Human Development*

Jonathan Graff, M.D., Ph.D., *Harvard University*

Kazuto Kajiwara, M.D., *Harvard Medical School*

Gail Pairitz-Jarvik, M.D., Ph.D., *University of Washington*

Immunology

Joan Butterton, M.D., *Massachusetts General Hospital*

Suzanne Conzen, M.D., *Dartmouth Medical School*

Michael Hagensee, M.D., Ph.D., *Fred Hutchinson Cancer Research Center*

Shan Lu, M.D., Ph.D., *University of Massachusetts Medical Center*

John Ogundipe, M.D., Ph.D., *Case Western Reserve University*

Peter Pertel, M.D., *Northwestern University*

Lalita Ramakrishnan, M.D., Ph.D., *Stanford University School of Medicine*

Dennis Walling, M.D., *University of North Carolina at Chapel Hill*

Wendy Ward, M.D., *University of California, San Francisco*

Neuroscience and Physiology

Robert Chow, M.D., Ph.D., *Max Planck Institute for Experimental Medicine, Germany*

Timothy Kamp, M.D., Ph.D., *The Johns Hopkins University School of Medicine*

Trevor Kilpatrick, M.D., Ph.D., *The Salk Institute for Biological Studies*

Maxine Lee-Mengel, M.D., *Julius Maximilians University, Germany*

Barry London, M.D., Ph.D., *Children's Hospital, Boston**

Pamela Sklar, M.D., Ph.D., *Columbia University College of Physicians and Surgeons**

David Standaert, M.D., Ph.D., *Massachusetts General Hospital*

Scott Turner, M.D., Ph.D., *University of Pennsylvania*

Zi-Jian Xu, M.D., Ph.D., *Stanford University School of Medicine**

*Awardees under this program who conduct their postdoctoral research in an Institute laboratory are appointed as Howard Hughes Medical Institute Associates.

Continuing Awards—Postdoctoral Research Fellowships for Physicians

Cell Biology and Regulation

Michael Apkon, M.D., Ph.D., *Yale University School of Medicine*

Lawrence Simeon Barak, M.D., Ph.D., *Duke University Medical Center*

Anastasia Golfinos Daifotis, M.D., *Yale University School of Medicine*

Roberta Ann Gottlieb, M.D., *University of California, San Diego*

Peter Steven Klein, M.D., Ph.D., *Harvard University*

Frederik Peter Lindberg, M.D., Ph.D.,
Washington University

Jason Drew Morrow, M.D., *Vanderbilt
University School of Medicine*

Louie Naumovski, M.D., Ph.D., *Stanford
University School of Medicine*

Thomas Anthony Rando, M.D., Ph.D., *Stanford
University School of Medicine*

James Paul Walsh, M.D., Ph.D., *University of
Washington**

Michael Alan Wood, M.D., *Dana-Farber Cancer
Institute*

Genetics

Mark Aaron Behlke, M.D., Ph.D., *Massachusetts
Institute of Technology**

Douglas Paul Clark, M.D., *University of
Pennsylvania*

James Philip Evans, M.D., Ph.D., *University of
Washington**

Robert Vito Farese, Jr., M.D., *J. David Gladstone
Institutes*

David Erich Fisher, M.D., Ph.D., *Massachusetts
Institute of Technology*

Lora Ann Hedrick, M.D., *The Johns Hopkins
University School of Medicine*

Henry Hung Hsu, M.D., *Stanford University
School of Medicine*

Clifford Arthur Lowell, M.D., Ph.D., *University
of California, San Francisco*

Craig Alan MacArthur, M.D., Ph.D., *Childrens
Hospital, Los Angeles*

Mia Michelle MacCollin, M.D., *Massachusetts
General Hospital*

Xavier S. Nassif, M.D., Ph.D., *Oregon Health
Sciences University*

Amy Lynn O'Donnell, M.D., *State University of
New York at Buffalo*

Warren Scott Pear, M.D., Ph.D., *Rockefeller
University*

Sharon Emma Plon, M.D., Ph.D., *Fred
Hutchinson Cancer Research Center*

Charles Lazelle Sawyers, M.D., *University of
California, Los Angeles**

Anthony Joseph Straceski, M.D., *Albert Einstein
College of Medicine of Yeshiva University*

Eric E. Turner, M.D., Ph.D., *University of
California, San Diego**

Mark William Walberg, M.D., Ph.D., *Stanford
University Medical Center*

Matthew K. Waldor, M.D., Ph.D., *Harvard
Medical School*

Rebecca Gray Wells, M.D., *Whitehead Institute
for Biomedical Research*

John Bernard Williams, M.D., Ph.D., *The Johns
Hopkins University**

Anthony Wynshaw-Boris, M.D., Ph.D., *Harvard
University**

Immunology

Richard Jerome Bram, M.D., Ph.D., *Stanford
University**

Yang-Xin Fu, M.D., Ph.D., *National Jewish
Center for Immunology and Respiratory
Medicine*

William Arthur Hagopian, M.D., Ph.D.,
University of Washington

Frank Davis Howard, M.D., Ph.D., *Dana-Farber
Cancer Institute*

Alan Ken Matsumoto, M.D., *The Johns Hopkins
University School of Medicine*

Elizabeth Jane McFarland, M.D., *University of
Colorado Health Sciences Center*

David Brian Roth, M.D., Ph.D., *National
Institutes of Health, National Institute of
Diabetes, Digestive, and Kidney Diseases*

Stacy Cheryl Smith, M.D., *Washington University*

Neuroscience

James Raymond Brorson, M.D., *The University of
Chicago*

Stephen Caldwell Cannon, M.D., Ph.D.,
*Massachusetts General Hospital**

Jonathan Alan Epstein, M.D., *Brigham and
Women's Hospital, Boston**

Michael Ralph Lauer, M.D., Ph.D., *Stanford
University**

Michael Neil Shadlen, M.D., Ph.D., *Stanford
University School of Medicine*

Morgan Hwa-Tze Sheng, M.D., *University of
California, San Francisco**

Hue-Teh Shih, M.D., *Baylor College of Medicine*

George Alexander West, M.D., Ph.D., *University
of Washington*

Mark E. von Zastrow, M.D., Ph.D., *Stanford
University**

Structural Biology

Michael Kenneth Gilson, M.D., Ph.D., *University
of Houston*

Irwin J. Kurland, M.D., *State University of New
York at Stony Brook*

Paul Thomas Wilson, M.D., Ph.D., *University of
California, San Francisco*

*Awardees under this program who conduct their
postdoctoral research in an Institute laboratory
are appointed as Howard Hughes Medical Insti-
tute Associates.*

UNDERGRADUATE SCIENCE EDUCATION

The Institute established the Undergraduate Biological Sciences Education Initiative to enhance undergraduate education and research in the biological sciences and the fields of chemistry, physics, and mathematics at academic institutions in the United States. Major goals include encouraging undergraduate students, particularly women and members of minority groups underrepresented in the sciences, to prepare for graduate studies and careers in biomedical education, research, or medical practice.

To achieve these goals, the initiative supports institutions in providing undergraduate research opportunities; broadening the academic base of biology through closer integration with chemistry, physics, and mathematics; enriching science faculties; enhancing the undergraduate research environment with modern equipment and laboratory renovations; and strengthening the ties between academic institutions and elementary, middle, and high schools and two- and four-year institutions to improve teaching in biology and other sciences.

Program awards. In 1988 the Institute awarded grants in the amount of $30.4 million to 44 private liberal arts colleges and public and private historically African-American institutions in an initial round of grants competition. The next year the Institute provided grant awards totaling $61 million to 51 research and doctorate-granting universities to enrich their undergraduate science programs. In a third round of grants competition, completed in 1991, the Institute made awards of $31.5 million to an additional 44 public and private liberal arts and comprehensive institutions, including a number of institutions with significant enrollments of underrepresented minority students. In 1992 the Institute awarded grants totaling $52.5 million to 42 research and doctorate-granting universities.

Since 1988 the Institute has provided a total of $175.5 million to 181 institutions for five-year grants to support undergraduate science education. Of that amount, approximately $60 million is being used at 170 institutions for programs to recruit and retain students in the sciences, especially those underrepresented in scientific fields, such as women, African-Americans, Hispanics, and Native Americans. The principal student activity supported under the program is undergraduate research, providing opportunities for students, many with no prior laboratory experience, to learn scientific concepts and techniques while assisting scientists in on- and off-campus laboratories. An additional $28 million is being used by 98 awardee institutions for science faculty development, including the appointment of new faculty members and programs to engage research faculty in undergraduate teaching.

Approximately $53 million has been directed to the development of science curricula and laboratories, enabling nearly all of the 181 awardee institutions to enhance the quality of instruction in the biological sciences and other disciplines as they relate to biology. Another $34 million is supporting precollege and outreach programs at 170 grantee colleges and universities to expand existing linkages and to develop new ones with precollege and other institutions.

To date the Institute's undergraduate awards have supported more than 7,800 opportunities for hands-on research by undergraduates, of whom 26% are students from minority groups and 54% are women. In addition, the program has supported the development of new research-oriented science curricula, including the acquisition of equipment and renovation of teaching laboratories. These curricula are in fields represented by the Institute's scientific programs, namely cell biology and regulation, genetics, and neuroscience, and in related fields such as biochemistry, molecular biology, and physiology. The undergraduate program has also supported the appointment of 114 science faculty members, of whom 16% are minorities and 46% are women. These appointments represent a range of disciplines in the biological sciences and the related areas of chemistry, physics, and mathematics.

About 5,500 precollege teachers and 16,000 students have participated in science outreach programs supported by the undergraduate grants program. Approximately 19% of these teachers and 66% of the students are members of minority groups underrepresented in science disciplines; 56% of the teachers and 57% of the students are female.

Program directors meetings. As part of its ongoing assessment endeavor, the Institute brings together the directors of undergraduate programs it supports. These meetings provide the Institute with the opportunity to learn more about the activities it has funded and to gain important background on the priorities and needs of undergraduate science education for future program development. To inform the scientific and educational communities of these activities, the Institute publishes proceedings and other reports, which are distributed nationally.

The initial meeting of the program directors in 1991 focused on undergraduate research and precollege and outreach activities funded by the Institute at the grantee institutions. The proceedings of this meeting and individual program descriptions are included in the Institute publication *Attracting*

Students to Science: Undergraduate and Precollege Programs, 1992.

The theme of the 1992 meeting was curriculum and laboratory development and undergraduate research. Presentations focused on undergraduate programs at the introductory, intermediate, and upper-division levels in such fields as biochemistry, cell and molecular biology, genetics, and neuroscience. Program directors also explored approaches to integrating biology teaching with other scientific fields and developing interdisciplinary laboratory courses, and emphasized hands-on research in the undergraduate curriculum. The reports from the meeting, *Enriching the Undergraduate Laboratory Experience* and the *1993 Undergraduate Program Directory*, will be published early in 1993.

New grants competition. Based on information collected through these assessment activities, the Institute announced a new phase of the undergraduate program for 1993. Support for undergraduate research, including opportunities for women and underrepresented minority students, remains as a central component. Activities to prepare students for laboratory research and enable them to present their findings will also be supported. In addition, support for precollege and outreach programs in the sciences will continue to be a priority. The new program will provide major support for the infrastructure of undergraduate teaching and research, including new equipment and laboratory renovations.

In 1992 the Institute invited 185 public and private comprehensive colleges and universities, liberal arts colleges, and schools of engineering and technology to submit grant proposals. These institutions were selected on the basis of their recent records of graduating students who go on to medical school or to earn doctorates in the biological sciences, chemistry, physics, or mathematics. Institutions receiving Institute awards in 1988 and 1991 and other institutions included in the above Carnegie Foundation classifications were invited to compete for four-year awards. A panel of scientists will evaluate the proposals, and approximately $27 million in grants will be announced in the summer of 1993.

New Awards—Undergraduate Biological Sciences Education

Arizona State University
Auburn University, Alabama
Boston University, Massachusetts
Brandeis University, Massachusetts
California Institute of Technology
Georgetown University, Washington, D.C.

Harvard University, Massachusetts
Illinois Institute of Technology
Iowa State University
Kansas State University
Marquette University, Wisconsin
Michigan State University
North Carolina State University
Oklahoma State University
Rutgers, the State University of New Jersey, Newark
State University of New York at Albany
State University of New York at Binghamton
Texas Tech University
University of California, Berkeley
University of California, Los Angeles
University of California, Santa Barbara
University of Cincinnati, Ohio
University of Delaware
University of Georgia
University of Hawaii at Manoa
University of Iowa
University of Kentucky
University of Maryland, College Park
University of Massachusetts at Amherst
University of Michigan–Ann Arbor
University of Nebraska–Lincoln
University of Nevada, Reno
University of New Mexico
University of Notre Dame, Indiana
University of Oregon
University of Pittsburgh, Pennsylvania
University of Rochester, New York
University of South Carolina–Columbia
Vanderbilt University, Tennessee
Washington State University
Washington University, Missouri
West Virginia University

Undergraduate Awards, 1988–1991

Allegheny College, Pennsylvania
Amherst College, Massachusetts
Antioch University, Ohio
Barnard College, New York
Bates College, Maine
Beloit College, Wisconsin
Brown University, Rhode Island
Bryn Mawr College, Pennsylvania
Bucknell University, Pennsylvania
California State University, Long Beach
California State University, Los Angeles
Calvin College, Michigan
Canisius College, New York
Carleton College, Minnesota
Carnegie Mellon University, Pennsylvania
Case Western Reserve University, Ohio

Centre College, Kentucky
City College of the City University of New York
City University of New York, Brooklyn College
City University of New York, Queens College
Clark College, Georgia
Colby College, Maine
Colgate University, New York
College of the Holy Cross, Massachusetts
College of William and Mary, Virginia
College of Wooster, Ohio
Colorado College
Colorado State University
Columbia University, New York
Cooper Union, New York
Cornell University, New York
Dartmouth College, New Hampshire
Davidson College, North Carolina
DePauw University, Indiana
Dillard University, Louisiana
Duke University, North Carolina
Earlham College, Indiana
Eckerd College, Florida
Emory University, Georgia
Fisk University, Tennessee
Fort Lewis College, Colorado
Franklin and Marshall College, Pennsylvania
Gettysburg College, Pennsylvania
Goucher College, Maryland
Grinnell College, Iowa
Hamilton College, New York
Hampshire College, Massachusetts
Hampton University, Virginia
Harvey Mudd College, California
Haverford College, Pennsylvania
Hiram College, Ohio
Hope College, Michigan
Howard University, Washington, D.C.
Illinois Benedictine College
Indiana University
Jackson State University, Mississippi
Johns Hopkins University, Maryland
Juniata College, Pennsylvania
Kenyon College, Ohio
King College, Tennessee
Knox College, Illinois
Lafayette College, Pennsylvania
Lawrence University, Wisconsin
Lehigh University, Pennsylvania
Lincoln University, Pennsylvania
Louisiana State University
Macalester College, Minnesota
Manhattan College, New York
Marlboro College, Vermont
Massachusetts Institute of Technology
Miami University, Ohio

Middlebury College, Vermont
Millsaps College, Mississippi
Morehouse College, Georgia
Morgan State University, Maryland
Mount Holyoke College, Massachusetts
Nebraska Wesleyan University
New York University
Oakwood College, Alabama
Oberlin College, Ohio
Occidental College, California
Ohio State University
Ohio Wesleyan University
Pennsylvania State University
Pomona College, California
Princeton University, New Jersey
Purdue University, Indiana
Reed College, Oregon
Rensselaer Polytechnic Institute, New York
Rhodes College, Tennessee
Rice University, Texas
Saint Olaf College, Minnesota
San Diego State University, California
Smith College, Massachusetts
Spelman College, Georgia
Stanford University, California
Stevens Institute of Technology, New York
Swarthmore College, Pennsylvania
Tufts University, Massachusetts
Tuskegee University, Alabama
Union College, New York
University of Arizona
University of California, Davis
University of California, Irvine
University of California, San Diego
University of California, Santa Cruz
University of Chicago, Illinois
University of Colorado
University of Illinois, Chicago
University of Illinois, Urbana-Champaign
University of Kansas
University of Minnesota–Twin Cities
University of Missouri–Columbia
University of North Carolina, Chapel Hill
University of Pennsylvania
University of Puerto Rico, Mayaguez
University of Puerto Rico, Rio Piedras
University of Scranton, Pennsylvania
University of the South, Tennessee
University of Southern California
University of Texas, Austin
University of Texas at San Antonio
University of Utah
University of Vermont
University of Virginia
University of Washington

University of Wisconsin–Madison
Ursinus College, Pennsylvania
Vassar College, New York
Villanova University, Pennsylvania
Wabash College, Indiana
Wake Forest University, North Carolina
Wayne State University, Michigan
Wellesley College, Massachusetts
Wesleyan University, Connecticut
Whitman College, Washington
Williams College, Massachusetts
Xavier University of Louisiana
Yale University, Connecticut

PRECOLLEGE AND PUBLIC SCIENCE EDUCATION
Precollege Science Education

In May 1991, the Institute announced its newest program in the area of elementary and secondary school education, a precollege science education initiative for museums. With a major focus on elementary school-age children, this initiative complements the outreach activities funded by the Institute through the undergraduate science education program, which have been geared primarily to secondary school students and teachers. The museums initiative was launched in recognition of the important role that museums have played in making science attractive and interesting to children and youth and in strengthening the science literacy of the general population. A total of $6.4 million in grants was awarded under this program in June 1992 to 29 institutions, including children's museums, natural history museums, and science museums and science and technology centers. Over the next five years, these awards will support early childhood and youth activities, curriculum enhancement, and teacher training, with special attention to minority and disadvantaged populations in rural and urban areas.

1992 Grant Awards to Museums

American Museum of Natural History, New York City, New York
Ann Arbor Hands-On Museum, Ann Arbor, Michigan
Buffalo Museum of Science, Buffalo, New York
Carter House Natural Science Museum, Redding, California
Children's Museum, St. Paul, Minnesota
Cleveland Children's Museum, Cleveland, Ohio
Cumberland Science Museum, Nashville, Tennessee
Discovery Center, Ft. Lauderdale, Florida
Discovery Place of Birmingham, Inc., Birmingham, Alabama
Fernbank Museums, Atlanta, Georgia

Franklin Institute, Philadelphia, Pennsylvania
High Desert Museum, Bend, Oregon
Imaginarium, Anchorage, Alaska
Lab 3000, Littleton, Colorado
Lawrence Hall of Science, Berkeley, California
Memphis Pink Palace Museum, Memphis, Tennessee
Montshire Museum of Science, Norwich, Vermont
Museum of Science and History, Jacksonville, Florida
Museum of Science and Industry, Chicago, Illinois
North Carolina Museum of Life and Science, Durham
North Museum, Lancaster, Pennsylvania
Oklahoma Museum of Natural History, Norman
Pacific Science Center, Seattle, Washington
Santa Fe Children's Museum, Santa Fe, New Mexico
Science Museum of Long Island, Manhasset, New York
Science Museum of Minnesota, St. Paul, Minnesota
Scotia Glenville Children's Museum, Scotia, New York
University of Nebraska State Museum, Lincoln
Virginia Tech Museum of Natural History, Blacksburg

Local Precollege Education Initiatives

In 1992 the Institute awarded three grants for the continuation of precollege life science education projects initiated over the last several years in the greater Washington, D.C., area, the local community of the Institute headquarters. A primary goal of these local science education grants is to increase the interest in science and science-oriented careers of girls and minorities underrepresented in the sciences.

Two of the 1992 grant awards link area high school students and teachers with biologists at the National Institutes of Health in Bethesda, where participants are involved in intensive research experiences for a full year. The Montgomery County Public Schools Educational Foundation, Inc., received a $150,000 grant in support of a third year of the student and teacher intern program that was initiated in 1990 as a pilot project with support from the Institute. The grant is providing 15 students and 3 teachers from Montgomery County high schools with the opportunity to work full time in the summer of 1992 and part time during the 1992–1993 school year.

A grant of $30,000 was also awarded in 1992 to the Foundation for Advanced Education in the Sciences at NIH in support of activities related to the student and teacher intern program. A major portion of the grant is for distribution of a videotape (developed with funds awarded by the Institute in 1991)

that tracks the progress of the student interns and how their perceptions of science and scientists change over the course of the internship.

A third local grant awarded by the Institute in 1992 will be used to initiate a program to train teachers on how to use the Carnegie Institution of Washington's First Light science curricula. First Light is a hands-on science program, developed in part with support from the Institute, for inner-city Washington, D.C., elementary school-age children. The $25,000 one-year grant from the Institute will support planning activities for the teacher training program.

HEALTH SCIENCES POLICY

In considering future grants program development, the Institute plans to explore possible initiatives in the general areas of health sciences policy and bioethics, addressing, for example, the issues of public understanding of science, the impact of technology on society, and the various roles of the public and private sectors in national and international policies related to science and technology.

In 1987 the Institute awarded a grant to the Institute of Medicine (IOM) of the National Academy of Sciences to develop studies and program activities that address a variety of topics in health sciences policy. Research briefings have been held and the proceedings published, highlighting areas of biomedical science that merit increased attention by researchers.

In addition, a series of workshops has been held by the IOM Committee on Technological Innovation in Medicine to identify mechanisms for facilitating the translation of basic biological discoveries into improvements in medical practice. A third subject covered by the IOM under the Institute grant concerns how decisions about science and health policy matters are affected by the level of public understanding of the science and technology involved. The case studies and the IOM committee's analysis of six major biomedical policy issues were published in June 1991, in a book entitled *Biomedical Politics*.

The studies and reports that result from the grant to the IOM will be used to help guide the Institute's development of grants initiatives in the areas of health sciences policy and bioethics.

INTERNATIONAL PROGRAM

The Office of Grants and Special Programs added a special international focus in 1991, with awards under the new International Research Scholars Program. In previous years there had not been a specific international program, although other grants programs served the international biomedical research community. The predoctoral and postdoctoral fellowships, for example, are open to foreign citizens for study in the United States, and U.S. citizens may study abroad. Furthermore, the Research Resources grants to the Cold Spring Harbor and Marine Biological Laboratories support their summer courses, which are attended by students and scientists from abroad as well as the United States.

International Research Scholars Program

In recognition of the contributions of scientists abroad to advances in biomedical science, the Institute launched a limited International Research Scholars Program on an experimental basis. The program is limited to specific selected countries and submission of applications is by invitation only. International Research Scholars Program awards provide support for the overall research program of promising scientists who have already made significant contributions to fundamental biomedical research. The awards are intended for outstanding scientists who are still developing, rather than for those in the later phases of a distinguished career. Scholars must hold appropriate full-time academic or research appointments at medical schools, universities, research institutions, or other not-for-profit scientific institutions in eligible countries.

Canada and Mexico, the immediate neighbors of the United States, were chosen as the two eligible countries for the first competition in the International Research Scholars Program. The current competition includes the United Kingdom, Australia, and New Zealand.

The Institute solicits nominations from Institute investigators and scientific advisors, as well as from scientific and academic leaders (such as deans and scientific directors at medical schools and research institutions) in countries eligible for awards. Eligible nominees rated most highly by biomedical scientists from universities and medical centers across the United States are invited to submit applications. The applications include a research plan that summarizes ongoing or planned work that Institute funding might enhance. These applications are evaluated by an external peer review panel. Based on the panel evaluations and the program goals, the Institute's management selects awards to be recommended to the Trustees for authorization for funding.

A total of $4.3 million was provided for research in Canada and Mexico during fiscal year 1992 under this program. There were 21 awards, including one group award, to the institutions of 14 scientists in Canada and 10 in Mexico. This included support for these scientists' equipment, supplies, travel, and

personnel such as graduate students, postdoctoral associates, and technicians. In addition, in recognition of the importance of the intellectual environment in which scholars conduct their research and the limited resources available in some circumstances, a portion of the awards in Mexico provide departmental shared resources that will enrich the general scientific environment. A listing of the scientists selected through this program appears in the introduction to this volume.

The International Research Scholars, among the foremost contributors to their fields, have participated with Institute investigators in the scientific meetings of the Institute. A summary of their grant-supported research is published each year in the Institute's annual *Research in Progress* and *Annual Scientific Report* and appears within this volume.

PROGRAM ASSESSMENT

Assessment within the grants program has two principal objectives: 1) to document and measure key outcomes of the Institute's various grants initiatives and 2) to assist the development of new initiatives through studies of national trends in areas such as science education, the national research enterprise, and public and private support for science.

The Institute's assessment program has focused initially on outcomes for the grants initiatives in graduate and undergraduate science education, largely through analysis of annual progress reports submitted by individual fellows and institutional grant recipients. Data from the reports are presented in *Grants for Science Education, 1992–1993*. Assessment programs are currently under development for several initiatives in the precollege education area.

In addition, several studies of national trends and conditions relevant to grants program planning and evaluation are under way or have been completed. In conjunction with these internal activities, the Institute is working with several federal agencies and private organizations to draw on existing national databases and to develop new ones. These will be used for long-term monitoring of the careers of Institute-supported fellows, the records of colleges and universities in graduating students who pursue careers in science and medicine, and analyses of other national trends relevant to support of scientific education and research.

One current project involving both assessment of an Institute fellowship program and studies at the national level concerns the education and career outcomes of physician-scientists. In order to measure the impact of Institute-sponsored programs for medical students and to gain a better understanding of the factors affecting career outcomes of physician-scientists, the Institute awarded a five-year grant to the Association of American Medical Colleges (AAMC) in 1989. The grant enables the AAMC to use existing national databases to track the educational progress and long-term careers of the Institute's fellows and scholars.

The project also tracks a number of other groups of M.D. graduates, for comparison with the Institute's fellow and scholar study groups. This project monitors annually a variety of educational and career outcomes for each group, beginning the year the M.D. is awarded, and will continue through some point beyond mid-career. This long-term tracking is designed to focus on involvement in research over time and at different career stages.

In addition to the primary objective of long-term tracking of the Institute's fellows and scholars, the AAMC study provides annual information on the national supply, demographic characteristics, and educational patterns of M.D./Ph.D.'s. Findings from this project, including data on annual enrollments in dual degree programs/numbers of earned doctorates among recent M.D.'s, the sequence and timing of the M.D. and Ph.D. degrees, and patterns of total educational indebtedness among recent medical school graduates, are presented in the annual volumes of *Grants for Science Education*.

Another national-level assessment project is an analysis of the undergraduate origins of recent matriculants to medical school and recent recipients of doctoral degrees in selected sciences, including the biological sciences. This study is based on data developed for the Institute's undergraduate science education program in order to evaluate colleges and universities for participation in the undergraduate grant competitions. The data examine the records for each of 1,413 universities and four-year colleges concerning undergraduate alumni having gone on recently to matriculate in medical school or to earn doctoral degrees in the sciences and mathematics. The study period is 1979 through 1988, with analyses of the absolute number and the proportion of baccalaureate graduates having gone on to these outcomes. Data for this study were provided by the Association of American Medical Colleges, the National Research Council of the National Academy of Sciences, and the U.S. Department of Education. A summary of these findings will be presented in *Grants for Science Education, 1992–1993*.

COMBINED ALPHABETICAL LISTING OF PUBLICATIONS
BY HHMI INVESTIGATORS
1991–1992

1. Abbott, C., **Blank, R.**, Eppig, J., Fiedoreck, F.T., **Friedman, J.M.**, Huppi, K., Jackson, I., Mock, B., Stoye, J., and Wiseman, R. 1992. Mouse chromosome 4 report. *Mamm Genome* 3:S55–S64.

2. Abeliovich, A., Gerber, D., Tanaka, O., Katsuki, M., Graybiel, A.M., and **Tonegawa, S.** 1992. On somatic recombination in the central nervous system of transgenic mice. *Science* 257:404–410.

3. Abmayr, S.M., Michelson, A.M., Corbin, V., **Young, M.**, and Maniatis, T. 1992. *nautilus*, a *Drosophila* member of the myogenic regulatory gene family. In *Neuromuscular Development and Disease* (Kelly, A.M., and Blau, H.M., Eds.). New York: Raven, pp 1–16.

4. Abraham, K.M., Levin, S.D., Cooke, M.P., and **Perlmutter, R.M.** 1991. Transgenic systems for the analysis of *src*-family kinase function. In *Advances in Regulation of Cell Growth: Genetic Approaches to Understanding Cell Activation*. New York: Raven, vol 2, pp 231–250.

5. Abrams, C.S., Ruggeri, Z.M., **Taub, R.**, Hoxie, J.A., Nagaswami, C., Weisel, J.W., and Shattil, S.J. 1992. Anti-idiotypic antibodies against an antibody to the platelet glycoprotein (GP) IIb-IIIa complex mimic GP IIb-IIIa by recognizing fibrinogen. *J Biol Chem* 267:2775–2785.

6. **Abrams, J.**, Lux, A., **Steller, H.**, and Kreiger, M. 1992. Macrophages in *Drosophila* embryos and L2 cells exhibit scavenger receptor-mediated endocytosis. *Proc Natl Acad Sci USA* 89:10375–10379.

7. Abrams, T.W., **Karl, K.A.**, and **Kandel, E.R.** 1991. Biochemical studies of stimulus convergence during classical conditioning in *Aplysia*: dual regulation of adenylate cyclase by Ca^{2+}/calmodulin and transmitter. *J Neurosci* 11:2655–2665.

8. Ackley, C., and **Cooper, M.D.** 1992. Characterization of a feline T-cell-specific monoclonal antibody reactive with a CD5-like molecule. *Am J Vet Res* 53:466–471.

9. Adams, B.S., **Leung, K.**, **Hanley, E.W.**, and **Nabel, G.J.** 1991. Cloning of RκB, a novel DNA-binding protein that recognizes the interleukin-2 receptor α chain κB site. *New Biol* 3:1063–1073.

10. Adams, E.M., Brown, M.C., **Nunge, M.**, Krych, M., and **Atkinson, J.P.** 1991. Contribution of the repeating domains of membrane cofactor protein (CD46) of the complement system to ligand binding and cofactor activity. *J Immunol* 147:3005–3011.

11. Adler, D.A., Bressler, S.L., Chapman, V.M., **Page, D.C.**, and Disteche, C.M. 1991. Inactivation of the *Zfx* gene on the mouse X chromosome. *Proc Natl Acad Sci USA* 88:4592–4595.

12. Albers, K., and **Fuchs, E.** 1992. The molecular biology of intermediate filament proteins. *Int Cytol Rev* 134:243–279.

13. **Albitar, M.**, Cash, F.E., Peschle, C., and **Liebhaber, S.A.** 1992. Developmental switch in the relative expression of the α1- and α2-globin genes in humans and in transgenic mice. *Blood* 79:2471–2474.

14. **Albitar, M.**, and **Liebhaber, S.A.** 1991. Regulation of the human embryonic and adult α-globin genes in transgenic mice. In *The Regulation of Hemoglobin Switching: Proceedings of the Seventh Conference on Hemoglobin Switching, Airlie, Virginia, September 8–11, 1990* (Stamatoyannopoulos, G., and Nienhuis, A.W., Eds.). Baltimore, MD: Johns Hopkins University Press, pp 55–69.

15. Albritton, L.A., Bowcock, A.M., Eddy, R.L., Morton, C.C., **Tseng, L.**, Farrer, L.A., Cavalli-Sforza, L.L., Shows, T.B., and **Cunningham, J.M.** 1992. The human cationic amino acid transporter (ATRC1): physical and genetic mapping to 13q12-q14. *Genomics* 12:430–434.

16. **Alexander-Bridges, M.**, Buggs, C., **Giere, L.**, **Denaro, M.**, Kahn, B., White, M., **Sukhatme, V.**, and **Nasrin, N.** 1992. Models of insulin action on metabolic and growth response genes. *Mol Cell Biochem* 109:99–105.

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HHMI employees are listed in boldface type.

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27. **Andersen, L.B.**, Wallace, M.R., Marchuk, D.A., Tavakkol, R., Mitchell, A.L., **Saulino, A.M.**, and **Collins, F.S.** 1991. A highly polymorphic cDNA probe in the NF1 gene. *Nucleic Acids Res* 19:3754.

28. **Anderson, D.J.** 1992. Molecular control of neural development. In *An Introduction to Molecular Neurobiology* (Hall, Z.W., Ed.). Sunderland, MA: Sinauer Associates, pp 355–387.

29. **Anderson, D.J.**, Carnahan, J.F., Michelsohn, A., and Patterson, P.H. 1991. Antibody markers identify a common progenitor to sympathetic neurons and chromaffin cells *in vivo* and reveal the timing of commitment to neuronal differentiation in the sympathoadrenal lineage. *J Neurosci* 11:3507–3519.

30. **Anderson, M.D.S.**, **Kunkel, L.M.**, and Khurana, T.S. 1992. Dystrophin mRNA in lyophilized tissue [letter]. *Nature* 335:778.

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1983	Richard K. Gershon, M.D.
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