ANNUAL SCIENTIFIC REPORT

1988–1989

HOWARD HUGHES MEDICAL INSTITUTE

Associate Director for Administration, NIH

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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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FOREWORD

The year 1988–89 has been one of great excitement for the Howard Hughes Medical Institute (HHMI), as the expansion in both the size and the scope of the Institute's activities has continued.

The largest and most long-standing of the Institute's programs, which has come to be known as the *First Program*, involves the conduct of biomedical research by its investigators in a large number of laboratories across the country, and is the principal subject of this report. The extensive and extremely high quality research accomplishments of the Institute scientists are described in detail later in this volume. There have been similarly auspicious developments in other programs, which I shall review briefly here.

The *Second Program* comprises several activities that are closely related to and support the First Program, including scientific meetings and workshops. This year the format of the Institute's regular scientific meetings was modified; instead of the meetings focusing on program areas such as Genetics or Cell Biology and Regulation, topical meetings were organized, e.g., "Receptors and Receptor Mechanisms" and "Regulation of Gene Expression." By all accounts these meetings were a great success, and in addition to their excellence and depth of coverage of important topics, they served to bring together investigators from different Institute programs and promote cross-fertilization of ideas and collaborations. The joint program with the National Institutes of Health (NIH) at the Cloister on the NIH campus, in which students come from many medical schools and spend a year as HHMI research scholars in NIH laboratories, has continued to prosper, attracting students who receive excellent research training and stimulating them to go on to research careers. HHMI has also maintained its support of databases concerned with the rapid accumulation of knowledge on the mapping of the human genome and of genes involved in human diseases. In the past year, support was initiated for a new relational genome database at The Johns Hopkins University School of Medicine.

The *Third Program* of the Institute, its grants program, which began only in 1987, completed its second full year on August 31, 1989. In this short time the grants program has grown spectacularly, providing vitally needed support for biomedical education. In the last fiscal year, approximately $42 million in grants were awarded, including new grants to 51 undergraduate colleges of research universities, to the Marine Biological Laboratories in Woods Hole, Massachusetts, for their specialized courses in the biological sciences, and to the Institute of Laboratory Animal Research. The first 47 awards of medical student research fellowships were made in a new program modeled on the highly successful program at the Cloister, except that the students spend a research year in their own or another medical school rather than going to the NIH. A new postdoctoral fellowship program designed to provide physicians with extensive training in basic science was also launched. The new and ongoing Grants and Special Programs activities are summarized more fully later in this volume.

One of the most exciting events of the past year was the beginning of the planning of a new headquarters and conference center for the Institute on a beautiful 22.5 acre plot of land in Chevy Chase, Maryland. An architectural firm, the Hillier Group from Princeton, New Jersey, was engaged in February 1989, and the design process was well along at the close of the fiscal year. This complex will serve as the headquarters for all the Institute's research and grants activities and as a scientific home for the Institute's investigators when they return for scientific meetings and visits. Completion of construction is anticipated in early 1992.

The increase in the scope of the Institute's activities is reflected in the size of the HHMI community. On August 31, 1989, there was a total of 1,793 employees nationwide, with a staff of 176 at the headquarters in Bethesda and 11 at the Cloister. A full description of the Institute's research activities and personnel follows.

Purnell W. Choppin, M.D.
President

INTRODUCTION

The accelerated growth of the research activities of the Institute that began in 1985 has been maintained through the present year and is projected to continue in the coming year. As of August 31, 1989, there were 1,552 individuals engaged in the conduct of research and research administration at the Institute's units and associated laboratories. It is their research efforts that are reported in this volume. The work was directed by 5 Senior Investigators, 91 Investigators, 36 Associate Investigators, and 60 Assistant Investigators and assisted by 49 Senior Associates and 424 postdoctoral Associates. The work of these scientists was aided by 449 research technicians, 94 research secretaries, and 344 others, including computer programmers, research specialists, laboratory aides, and managers of administrative services with their associated office staffs. Also working in the Institute's laboratories were 363 graduate students and 471 postdoctoral fellows, who contributed to the research of the HHMI Investigators but were not employees of the Institute.

LOCATIONS

By the end of August 1989, laboratories of the Howard Hughes Medical Institute were located at 37 sites in 30 cities in the United States, as listed elsewhere in this report. New or expanded research facilities were completed at the University of Michigan, the University of Pennsylvania, and Stanford University. Also under construction are laboratories at Children's Hospital in Boston, the University of California at Los Angeles and at San Diego, Yale University, and the University of Utah.

The Institute continues to expand its support of selected individual investigators. A further 10 such investigators were added this year, bringing the total in this category to 25 and extending the Institute's activities to four new sites. Those appointed were Assistant Investigators David J. Anderson, Ph.D., Pamela J. Bjorkman, Ph.D., and Paul W. Sternberg, Ph.D., at the California Institute of Technology; Investigators John E. Donelson, Ph.D., and Michael J. Welsh, M.D., at the University of Iowa; Investigators Thomas E. Shenk, Ph.D., and Shirley M. Tilghman, Ph.D., at Princeton University; and Investigators Christopher Miller, Ph.D., and Michael Rosbash, Ph.D., at Brandeis University. A second scientist, Associate Investigator Matthew P. Scott, Ph.D., was appointed at the University of Colorado. Further appointments in this category will be made in the coming year, so as to expand the Institute's presence at more institutions and to identify more promising new scientists at the Assistant Investigator level.

INVESTIGATORS

The following scientists were appointed to or promoted within the Institute's research staff:

Investigators:

- Joan S. Brugge, Ph.D., University of Pennsylvania School of Medicine
- John E. Donelson, Ph.D., University of Iowa
- Uta Francke, M.D., Stanford University Medical Center
- H. Ronald Kaback, M.D., University of California, Los Angeles
- Susan L. Lindquist, Ph.D., The University of Chicago
- Christopher Miller, Ph.D., Brandeis University
- Michael Rosbash, Ph.D., Brandeis University
- John W. Sedat, Ph.D., University of California, San Francisco
- Thomas E. Shenk, Ph.D., Princeton University
- Paul B. Sigler, M.D., Ph.D., Yale University
- Shirley M. Tilghman, Ph.D., Princeton University
- Roger Y. Tsien, Ph.D., University of California, San Diego
- Michael J. Welsh, M.D., University of Iowa
- Michael W. Young, Ph.D., The Rockefeller University

Associate Investigators:

- David A. Agard, Ph.D., University of California, San Francisco
- Francis S. Collins, M.D., Ph.D., University of Michigan
- Elaine V. Fuchs, Ph.D., The University of Chicago
- Randall R. Reed, Ph.D., The Johns Hopkins University
- Matthew P. Scott, Ph.D., University of Colorado
- Steven A. Siegelbaum, Ph.D., Columbia University
- Thomas C. Südhof, M.D., University of Texas Southwestern Medical Center at Dallas
- Arthur Weiss, M.D., Ph.D., University of California, San Francisco

Assistant Investigators:

- David J. Anderson, Ph.D., California Institute of Technology

Philip A. Beachy, Ph.D., The Johns Hopkins University

Pamela J. Bjorkman, Ph.D., California Institute of Technology

Rudolf Grosschedl, Ph.D., University of California, San Francisco

Harinder Singh, Ph.D., The University of Chicago

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

James M. Wilson, M.D., Ph.D., University of Michigan

AWARDS AND RECOGNITIONS

Especially striking is the recognition accorded to investigators of the Institute by their scientific peers through their nominations to prestigious professional societies; their appointments to local, national, and international advisory groups and review panels; and their work on editorial boards. As a group they remain dedicated to service to their institutions and the scientific community, as well as to their scientific research. One measure of the latter is the fact that during the period covered by this report, HHMI investigators published more than 1,500 original scientific papers and reviews of their work. They have presented prestigious, named lectures at institutions around the world, and certain aspects of their research have been supported through such special research grant awards as the Javits, MERIT, and Outstanding Investigator Grant awards of the National Institutes of Health. The list of honors they have been awarded is lengthy, and only a selection can be presented in this report.

Investigator Johann Deisenhofer shared the 1988 Nobel Prize in Chemistry with his German colleagues Drs. R. Huber and H. Michel. Investigators Ronald M. Evans, John W. Kappler, Philippa Marrack, and Allan C. Spradling were elected to the National Academy of Sciences. Investigator Richard O. Hynes was elected to the Royal Society of London. Investigator Thomas R. Cech was awarded the Louisa Gross Horvitz Prize from Columbia University and the Lewis Rosenstiel Award from Brandeis University and was among three who shared the 1988 Albert Lasker Basic Medical Research Award. Investigator Richard Axel received the Richard Lounsbery Award of the National Academy of Sciences. Associate Investigator Graeme I. Bell received the 1989 Mary Jane Kugel Award from the Juvenile Diabetes Foundation International. The American Federation for Clinical Research presented its prestigious Young Investigator Award to Investigators Perry J. Blackshear and Lewis T. Williams. Investigator Günter Blobel received the Waterford Award. The Glaxo Discovery Award was presented to Investigator Jackie D. Corbin. Investigator Ronald M. Evans received the Van Meter/Rorer Pharmaceuticals Prize. The American Association of Anatomists presented the R.R. Bensely Award to Associate Investigator Elaine V. Fuchs. Assistant Investigator Kenneth J. Hardy received the Scientific Excellence Award of the International Society for Interferon Research. Investigators Lily and Yuh Nung Jan were recipients of the W. Alden Spencer Award and Lectureship of the Columbia University College of Physicians and Surgeons, and Dr. Lily Jan also received the Fourteenth Annual Mildred Trotter Award from Washington University School of Medicine. Investigator Yuet Wai Kan received the Premio Internazionale San Remo per le Ricerche Genetiche.

Senior Investigator Eric R. Kandel received the Distinguished Service Award of the American Psychiatric Association, the Award in Basic Science of the American College of Physicians, and the Robert J. and Claire Pasarow Foundation Award in Neuroscience. The 3M Life Sciences Award was presented to Senior Investigator Edwin G. Krebs. The Royal Society Wellcome Foundation Prize, the Warren Alpert Foundation Prize, and the Passano Foundation Young Scientist Award were presented to Associate Investigator Louis M. Kunkel, who also received the Distinguished Alumni Award from Gettysburg College.

The Milken Family Medical Foundation presented the Basic Cancer Research Award to Senior Investigator Philip Leder and an award to Investigator Robert Tjian. Dr. Leder also received the V.D. Mattia Award of the Roche Institute of Molecular Biology. Investigator Steven L. McKnight was the recipient of the Eli Lilly and the Newcomb Cleveland Awards. Assistant Investigator Jeremy Nathans received the Wilson S. Stone Award of the M.D. Anderson Cancer Center and the 1988 Distinguished Young Scientist Award of the Maryland Academy of Sciences. Investigator Allan C. Spradling received the 1989 Medal of the Genetics Society of America. The Mount Sinai School of Medicine awarded Investigator Joan A. Steitz a D.Sc. (Hon.) degree. Dr. Steitz also received the Dickson Prize for Science from Carnegie-Mellon University. Investigator Susumu Tonegawa received the Kihara Prize of the Japanese Genetics Society and the Distinguished Investigator Award of the American College of Rheumatology. Associate Investigator Francis S. Collins was honored by the Michigan Chapter of the National Neurofibromatosis Foundation with its award for outstanding contributions to research on

neurofibromatosis. Investigator Charles T. Esmon received the Merrick Distinguished Scientist Award of the Oklahoma Medical Research Foundation. The Frank E. Trobaugh Hematology Young Investigator Award was presented to Assistant Investigator David Ginsburg by the Midwest Blood Club. Associate Investigator Gary K. Schoolnik was presented with the 1989 Kaiser Family Foundation Award for Preclinical Teaching at Stanford University Medical School. Assistant Investigator Craig B. Thompson received the Jerome W. Conn Award for Distinguished Research by a Junior Faculty Member at the University of Michigan Medical Center.

In 1989 Investigator Shirley M. Tilghman served as Chairman of the Board of Scientific Overseers of The Jackson Laboratory and Investigator Stuart H. Orkin served as President of the American Society of Clinical Investigation. Investigator Larry J. Shapiro was elected President of the American Board of Genetics, of the Western Society for Pediatric Research, and of the International Pediatric Research Foundation. Investigator Robert J. Lefkowitz is Treasurer of the American Association of Physicians. Presidents-elect of their respective societies are Investigator Günter Blobel, American Society for Cell Biology; Investigator C. Thomas Caskey, American Society of Human Genetics; Investigator Allan C. Spradling, Society for Developmental Biology; and Investigator Larry J. Shapiro, Society for Pediatric Research.

W. Maxwell Cowan, M.D., Ph.D.
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Lewis, Dorothy E., Ph.D.
Littman, Dan R., M.D., Ph.D.
Lowe, John B., M.D.
Mowbray, Sherry L., Ph.D.
Nabel, Gary J., M.D., Ph.D.
Nathans, Jeremy, M.D., Ph.D.
Overbeek, Paul A., Ph.D.
Parker, Keith L., M.D., Ph.D.
Payan, Donald G., M.D.
Perrimon, Norbert, Ph.D.
Peterlin, B. Matija, M.D.
Reeders, Stephen T., M.D.
Sakonju, Shigeru, Ph.D.
Singh, Harinder, Ph.D.
Slaughter, Clive A., Ph.D.
Soriano, Philippe M., Ph.D.
Sprang, Steven R., Ph.D.
Sternberg, Paul W., Ph.D.
Struhl, Gary, Ph.D.
Sukhatme, Vikas P., M.D., Ph.D.
Thompson, Craig B., M.D.
Thummel, Carl S., Ph.D.
Van Dop, Cornelis, M.D., Ph.D.
Williams, David A., M.D.
Wilson, James M., M.D., Ph.D.
Yellen, Gary I., Ph.D.

LOCATIONS OF HOWARD HUGHES MEDICAL INSTITUTE LABORATORIES

Alabama	University of Alabama at Birmingham
California	California Institute of Technology and associated hospitals, Pasadena 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 UCSD Medical Center, and The Salk Institute for Biological Studies University of California, San Francisco, 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
Connecticut	Yale University and associated hospitals, New Haven
Illinois	The University of Chicago and The University of Chicago Hospitals
Iowa	University of Iowa and associated hospitals, Iowa City
Maryland	The Carnegie Institution of Washington and The Johns Hopkins Hospital, Baltimore The Johns Hopkins University and Hospital, Baltimore
Massachusetts	Brandeis University, Waltham Harvard University consortium: Brigham and Women's Hospital, Boston 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
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	Columbia University 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
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 University of Texas Southwestern Medical Center at Dallas and associated hospitals
Utah	University of Utah and University of Utah Medical Center, Salt Lake City
Washington	University of Washington and associated hospitals, Seattle

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OTHER INSTITUTE FACILITIES

Connecticut	Human Genome Mapping Library, New Haven
Maryland	HHMI-NIH Cloister at the Mary Woodard Lasker Center for Science and Education on the NIH campus, Bethesda
New York	Synchrotron Beam Line, Brookhaven National Laboratory (under construction)

HOWARD HUGHES MEDICAL INSTITUTE
SCIENTIFIC MEETINGS 1988–1989

February 26–March 1, 1989	Receptors and Receptor Mechanisms
March 12–15, 1989	Intracellular Communication and Signaling
April 9–12, 1989	Regulation of Gene Expression

SCIENTIFIC CONFERENCE

May 14–17, 1989	Workshop on cAMP-dependent Protein Kinase Dr. Jackie D. Corbin, organizer

I. CELL BIOLOGY AND REGULATION

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I. PROGRAM IN CELL BIOLOGY AND REGULATION

The Program in Cell Biology and Regulation is a continuation of the Institute's oldest research program, formed in 1976 under the title "Metabolic Regulation." In 1985 the program was renamed to reflect the Institute's growing activity in the area of molecular cell biology. Investigators working in this area are located at the University of Michigan, The Carnegie Institution of Washington at Baltimore, the Brigham and Women's Hospital in Boston, the Massachusetts General Hospital, the Massachusetts Institute of Technology, The University of Chicago, the University of Texas Southwestern Medical Center at Dallas, Duke University, the University of Iowa, the University of California at both Los Angeles and San Francisco, Vanderbilt University, The Rockefeller University, the Oklahoma Medical Research Foundation, Stanford University, the University of Pennsylvania, Princeton University, Washington University in St. Louis, and the University of Washington. The studies being conducted under this program largely concern the metabolic pathways and molecular processes that are basic to the growth and differentiation of cells, communication within and between cells, cell-substrate relationships, and some of the disorders that occur when these processes are perturbed.

Senior Investigator Donald F. Steiner, M.D. (The University of Chicago) and his colleagues are continuing their studies of the genetic and molecular mechanisms underlying the production and action of insulin and related regulatory hormones produced in the islets of Langerhans of the pancreas. The biological roles of various precursor peptides, such as proinsulin, and the cell biological and enzymatic mechanisms that lead to their transformation into active hormones or neuropeptides are also being studied. Their long-term goals are to develop information that will increase understanding of the production of insulin and its mode of action and of the growth and development of islet tissue, with a view to providing insights into the causes of human diabetes mellitus.

Investigator Joseph Avruch, M.D. (Massachusetts General Hospital) continues to examine the sequence of reactions by which insulin modifies cell function. The most significant findings in the past year relate to the purification of two proteins that are among the initial targets of the activated insulin receptor.

The research of Investigator Perry J. Blackshear, M.D., D.Phil. (Duke University) is also focused on the biochemical reactions that occur when insulin binds to its receptor in the membrane cells and on some of the later molecular changes that this event initiates. One of the earliest such changes is the rapid turning on of the c-*fos* proto-oncogene. This response to insulin appears to involve a discrete segment of the c-*fos* gene. Dr. Blackshear's laboratory is also trying to determine how insulin modifies the ability of certain proteins to bind to DNA and, in this way, to redirect the metabolic reactions of the responding cells. A more complete understanding of the pathways involved should help to elucidate the process of normal cell growth, and such abnormalities as obesity and adult-onset diabetes.

In the absence of insulin there is a marked decrease in the tissue content of a number of rate-limiting enzymes that regulate the uptake and utilization of glucose. The laboratory of Assistant Investigator Maria C. Alexander-Bridges, M.D., Ph.D. (Massachusetts General Hospital) has defined two cis-acting sequences in the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene that together increase the inductive effect of insulin on the transcription of the GAPDH gene. Insulin-sensitive DNA-binding proteins interact with these sequences in fat and liver cells that store glucose as glycogen. Through her continuing studies Dr. Alexander-Bridges hopes to define the mechanisms by which insulin potentiates energy storage and to determine how the regulation of the GAPDH gene is altered in the diabetic state.

The laboratory of Investigator William W. Chin, M.D. (Brigham and Women's Hospital) is exploring the molecular mechanisms involved in the hormonal regulation of gene expression. In the past year, their efforts have been focused largely on the thyroid hormone and the sex steroids that respectively regulate the genes for thyrotropin and gonadotropin. Putative cis-acting DNA elements have been identified that appear to be critical for the response of these genes. In addition, an analysis of the molecular genetics of the various thyroid hormone receptor forms has demonstrated considerable heterogeneity in their structure and function. The goal is to define more precisely the structure of the hormone receptors and their interactions with the DNA regulatory regions responsible for both the negative and positive regulation of the responsive genes.

Two critical steps in thyroid hormone action have

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been investigated by the laboratory of Investigator P. Reed Larsen, M.D. (Brigham and Women's Hospital). The first is the process by which thyroxine, the prohormone secreted by the thyroid gland, is activated. This apparently simple reaction, the loss of an iodine atom, is tightly controlled by at least two enzymes that function to ensure adequate supplies of the active hormone under various circumstances. In the past year considerable progress has been made in the analysis of the structure of these enzymes. The second facet of Dr. Larsen's studies has been the identification of the thyroid-responsive sequences in the promoter region of genes that allow them to respond to thyroid hormone. A repeated pattern of three nearly identical segments of six specific nucleotides is required for a gene to be responsive to thyroid hormone. Surprisingly, at least some thyroid hormone–responsive genes cannot be expressed at maximum rates. This important observation may increase understanding of the role of thyroid hormone in the regulation of many basic metabolic processes in health and disease.

Investigator Joel F. Habener, M.D. (Massachusetts General Hospital) and his colleagues are studying the molecular mechanisms responsible for the regulation and cell-specific expression of genes encoding polypeptide hormones. Their work has led to the hypothesis that the combinatorial interactions of several DNA sequence elements and DNA-binding proteins result in the selective activation of specific genes in phenotypically distinct cells. Their current work is focused on the cell-specific and cAMP-mediated activation of the somatostatin and gonadotropin subunit genes and the cell-specific expression of the glucagon and angiotensinogen genes that are expressed in the pancreas and liver. Ultimately they hope to isolate and characterize structurally the cAMP-responsive DNA-binding phosphoproteins and to analyze the molecular interactions of these proteins with the specific DNA sequence elements.

Steroid hormones play essential roles in salt and water balance, in carbohydrate metabolism, and in reproduction. Their biosynthesis requires the action of a related group of enzymes that convert cholesterol to biologically active steroids. The laboratory of Assistant Investigator Keith L. Parker, M.D., Ph.D. (Duke University) is studying the factors that regulate the production of these steroidogenic enzymes in the adrenal gland. So far they have been able to define some of the DNA regulatory elements and the proteins that interact with them to control the expression of these enzymes. Efforts to purify the proteins that are most important for the regulated expression of the sequences are under way.

Investigator Robert J. Lefkowitz, M.D. (Duke University) reports the following accomplishments over the past year: 1) the delineation of the structural basis of β-adrenergic receptor function by a combination of approaches, including site-directed mutagenesis and the creation of chimeric or hybrid receptors; 2) the demonstration that the function of the β-adrenergic receptor is regulated by phosphorylation by the cAMP-dependent protein kinase and the β-adrenergic receptor kinase; 3) the successful cloning of the cDNA for the β-adrenergic receptor kinase, which appears to be the first sequenced member of a multigene family of receptor kinases that may have broad regulatory significance; and 4) the cloning of the genes for several new members of the adrenergic receptor family, in particular two new α_1-adrenergic receptor subtypes. These last studies suggest that the adrenergic receptor family is more heterogeneous than had previously been suspected and open the possibility of developing new, more-selective clinical therapeutic agents.

Protein traffic across distinct cellular membranes occurs by a sort of biological zip code system. The recognition of the various signal sequences that constitute the zip codes requires the intervention of complex cellular machineries, referred to as translocons. Each translocon is composed of at least four entities: 1) a soluble signal recognition factor (SRF) that is specific for each translocon, 2) a membrane-bound homing receptor specific for each SRF, 3) a channel in the membrane that is opened upon presentation of a translocon-specific signal sequence and is closed after a single protein is translocated, and 4) a translocon-specific signal peptidase that removes the signal sequences. The laboratory of Investigator Günter Blobel, M.D., Ph.D. (The Rockefeller University) has been investigating four different translocons. In addition he and his colleagues have continued their studies of the nuclear pore complexes and the lamina-associated components that are key structures involved in the functional organization of the genome.

Investigator John A. Glomset, M.D. (University of Washington) reports that research done in collaboration with Dr. Andreas Habenicht (University of Heidelberg) has identified a new role for plasma low-density lipoproteins: namely, delivery of the polyunsaturated fatty acid arachidonic acid to replicating cells. This role was shown to have a critical effect on the formation of two important regulatory

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molecules, prostaglandin E_2 and prostacyclin. Collaborative studies with Dr. Michael Gelb (University of Washington) have led to the identification of a new type of protein modification in animal cells. Lamin B, a nuclear protein from human cancer cells, was shown to contain a farnesyl group. Experiments are under way to explore the potential role of this modification in mediating the attachment of lamin B to the nuclear membrane.

The laboratory of Investigator Vann Bennett, M.D., Ph.D. (Duke University) is engaged in studies on ankyrin, a versatile family of proteins that participate in the placement of a variety of membrane proteins in specialized domains of the plasma membrane. The ankyrin family includes members with distinct localization and membrane receptors. One isoform of ankyrin found in brain is confined to the nodes of Ranvier and may play a role in the localization of voltage-dependent sodium channels to this region—an essential requirement for impulse conduction in myelinated nerve fibers. The diversity of ankyrins results in part from multiple genes, two of which have been cloned from human brain, and in part from alternative splicing of mRNA. The recognition site of ankyrin for one membrane protein has been narrowed to a region containing an evolutionarily ancient sequence motif also found in certain yeast and *Drosophila* proteins that are involved in the regulation of development.

Cells in the body are attached to adhesive proteins through cell surface receptors called integrins. This attachment is vital for proper cellular organization, structure, and metabolism and plays an important role in embryogenesis. The laboratory of Investigator Richard O. Hynes, Ph.D. (Massachusetts Institute of Technology) is involved in studies of several of the adhesive proteins, especially the fibronectins. Progress is reported in understanding 1) alterations of fibronectins and integrins in tumor cells, 2) the involvement of certain integrins in hemostasis and thrombosis, and 3) alterations in fibronectins during wound healing. The molecular details of these proteins and their roles in normal physiology and various pathological conditions continue to be examined.

The product of one proto-oncogene, platelet-derived growth factor (PDGF), stimulates cell proliferation by first binding to its receptor on the cell surface. Investigator Lewis T. Williams, M.D., Ph.D. (University of California at San Francisco) and his colleagues have studied the mechanism by which the surface receptor sends signals to the nucleus of the cell. This year they identified several of the signaling molecules with which the receptor interacts directly. These signaling enzymes include Raf-1, phosphatidylinositol kinase, and phospholipase C. For the first time this group has been able to use highly purified components to reconstruct the interactions between the receptor and the signaling molecules in cell-free systems.

Another polypeptide growth factor that triggers cell division by binding to a specific cell surface receptor and activating its tyrosine-specific protein kinase activity is studied by Investigator Charles J. Sherr, M.D., Ph.D. (St. Jude Children's Research Hospital). The growth factor in question is colony-stimulating factor-1 (CSF-1), whose receptor is encoded by a gene genetically linked to the gene for the PDGF receptor, the two genes having arisen by duplication and subsequent evolutionary divergence. Specific mutations in the CSF-1 receptor gene (c-*fms* proto-oncogene) were found to activate its kinase activity in the absence of CSF-1, thereby providing sustained signals for cell growth that lead to tumor formation. Because expression of the CSF-1 receptor is normally restricted to blood monocytes and tissue macrophages, such mutations might etiologically contribute to the development of myeloid leukemia.

Associate Investigator Linda J. Pike, Ph.D. (Washington University) is also interested in the mechanism of action of growth factors. Work in her laboratory has 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 recombinant DNA technology is being used to clone the enzyme. Other studies have shown that prolonged treatment of cells with EGF renders them insensitive to further stimulation by the 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, which may account for the inability of the cells to respond to the growth factor.

Investigator Mary-Jane Gething, Ph.D. (University of Texas Southwestern Medical Center at Dallas) and her colleagues study the molecular genetics of membrane and secretory proteins. Experiments focus on three proteins: 1) the hemagglutinin of influenza virus, which is being utilized as a marker molecule for specific cell populations in transgenic mice produced in an attempt to establish an animal

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model for type I diabetes; 2) human tissue-type plasminogen activator, which is being studied by oligonucleotide-directed mutagenesis to analyze the structure-function relationships of the individual domains of the molecule; and 3) BiP, a luminal protein of the endoplasmic reticulum that appears to be involved in the initial mobilization of proteins that traverse the secretory pathway.

Assistant Investigator Leland Ellis, Ph.D. (University of Texas Southwestern Medical Center at Dallas) and his colleagues are interested in the structure and function of those components of the plasma membrane that initiate the response of cells to extracellular stimuli, especially cell surface receptors. The insulin receptor comprises two large, soluble, functional domains connected by a single membrane-spanning domain. The two domains act in concert upon ligand binding (transmembrane signaling) to initiate the insulin response in cells, but each is also capable of autonomous function (ligand binding and protein tyrosine kinase activity, respectively). In collaborative studies with Dr. Robert E. Hammer (HHMI, University of Texas Southwestern Medical Center at Dallas), transgenic mice that express wild-type or altered forms of the receptor are being utilized to explore insulin receptor structure and function in the context of the intact animal, while the expression of soluble derivatives of each of the two major functional domains of the receptor facilitates the analysis of their biochemical and biophysical properties. Biochemical, molecular genetic, and transgenic approaches are also being employed to identify and study growth cone membrane-associated proteins in an effort to understand how growing nerve cells explore and respond to the complex extracellular microenvironment encountered during development of the nervous system.

Gene expression in animal cells is a complex process involving multiple, distinct steps. Expression of individual genes can be controlled at one or more of these steps, including messenger RNA synthesis, RNA processing, RNA transport, RNA half-life, protein synthesis, and protein half-life. The laboratory of Investigator Thomas Shenk, Ph.D. (Princeton University) uses a human DNA tumor virus, adenovirus, as a model to study the control of gene expression in animal cells. During the past year his laboratory has focused on the identification and characterization of cellular factors that mediate and regulate the synthesis of adenovirus mRNAs.

Transcriptional regulation in eukaryotic cells is primarily mediated through protein:DNA complexes. These complexes can form on DNA either close to the promoters for specific genes or at very distant sites, termed *enhancers*. In either case, the underlying DNA sequence encodes an ordered series of recognition sites for sequence-specific DNA-binding proteins. The appropriate amalgamation of binding sites somehow dictates when and where a gene is to be transcribed. Investigator Steven L. McKnight, Ph.D. (The Carnegie Institution of Washington) and his colleagues have used molecular genetic techniques to define the minimal DNA sequence elements from which enhancers and promoters are built and have used biochemical techniques to purify and study proteins that recognize these sequence elements.

The laboratory of Associate Investigator Nathaniel Heintz, Ph.D. (The Rockefeller University) is also interested in understanding the molecular events that lead to the expression of specific genes at particular times during the growth of mammalian cells. Efforts during the past year have resulted in the identification of several molecules that participate directly in the regulation of histone genes, whose activation occurs at a specific time during the cell growth cycle. Characterization of these molecules has led to the realization that a common mechanism may exist for activation of all macromolecular synthesis during the S phase of the cell cycle. Elucidation of this common mechanism may lead to some fundamental insight into the regulation of cell growth. Dr. Heintz's laboratory has also initiated a variety of approaches toward isolating genes that are involved in the development of the mammalian cerebellum.

Associate Investigator Elaine Fuchs, Ph.D. (The University of Chicago) and her colleagues seek to understand the molecular mechanisms that underlie growth, differentiation, and development in human epithelia, primarily the epidermis and its appendages. In the past year they have introduced a human keratin gene into mice and showed that it is properly regulated in skin cells. This approach is an important step in specifically targeting genes to skin. A second accomplishment was the demonstration that vitamin A inhibits abnormal differentiation in squamous cell carcinoma cells. Although this inhibition is encouraging, retinoids also increase proliferation of epidermal cultures, a potentially serious side effect for long-term retinoid treatments of various skin diseases. The laboratory also has engineered mutations in keratins and showed that they have a dominant effect when made by epidermal cells in culture. This suggests that naturally occur-

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ring mutations in keratins may lead to genetic skin disease.

Senior Investigator Edwin G. Krebs, M.D. (University of Washington) and his colleagues are interested in the molecular mechanisms involved in transmitting hormonal and growth factor signals within the cell. One of the major mechanisms that nature has employed for this purpose is the reversible modification of existing cellular proteins by phosphorylation and dephosphorylation. These modification reactions are catalyzed by enzymes referred to as protein kinases (phosphorylation steps) and protein phosphatases (dephosphorylation steps). This laboratory is determining how the kinases and phosphatases are regulated by signals that impinge on the cell. By this means it may become possible to modify growth factor and hormone responses with drugs so that abnormalities in the signaling process can be corrected.

Phosphorylation of rate-limiting proteins on serine and threonine residues by protein kinases is a common cellular mechanism for modulating physiological processes. In the brain, calcium-dependent protein kinases are important in coordinating the changes in intracellular calcium that occur with neuronal activity. The laboratory of Investigator Thomas R. Soderling, Ph.D. (Vanderbilt University) has elucidated regulatory mechanisms for an abundant multifunctional calcium-dependent protein kinase of brain. These mechanisms may prove to be important for altering and prolonging synaptic events in the nervous system.

The laboratory of Investigator Jackie D. Corbin, Ph.D. (Vanderbilt University) has studied the kinetics of the two cAMP sites of a fungal cAMP-dependent protein kinase and found them to be similar, but not identical, to those of the mammalian enzyme. Mammalian cGMP-dependent protein kinase has cGMP sites that are structurally similar to the cAMP sites of cAMP-dependent protein kinase but differ by one amino acid. Either of these two isolated kinases can function as a single protein chain, even though the natural mammalian enzymes have more than one chain. Cellular cAMP-dependent protein kinase regulates its own activation by stimulating, through phosphorylation, an enzyme that degrades cAMP.

In the laboratory of Investigator David L. Garbers, Ph.D. (Vanderbilt University) research on mechanisms of signaling in the spermatozoon resulted in the identification of the enzyme guanylate cyclase as a cell surface receptor. The cloning of the mRNA encoding this enzyme from mammalian tissues and the subsequent expression of guanylate cyclase in cultured cells demonstrated that it is a cell surface receptor for atrial natriuretic peptides. This enzyme receptor resembles the protein tyrosine kinase receptors by virtue of its possessing a single transmembrane domain and a protein kinase-like domain.

Eukaryotic cells respond to a diversity of physiological and pharmacological stimuli by molecular mechanisms that include transient rises in the concentration of calcium inside the cell. Regardless of their source, these changes in calcium concentration are transduced into a biological response through the interaction with calmodulin, a ubiquitous eukaryotic protein that has multiple biological roles. It is the goal of the research program in the laboratory of Investigator D. Martin Watterson, Ph.D. (Vanderbilt University) to understand the basic molecular mechanisms by which calmodulin is able to convert these small changes in calcium ion concentration into specific biological responses. Interdisciplinary studies employing biophysical methods and recombinant DNA technologies have led to a general model of how calcium signals are converted by calmodulin into a biological response that has provided a rational basis for the design and production of new chimeric proteins and a mechanistic interpretation of how inherited mutations of genes encoding calmodulin or a calmodulin-regulated enzyme might bring about selective nonlethal disorders.

Calcium-dependent hormones act by binding to specific cell surface receptors and stimulating the breakdown of certain membrane lipids to generate signaling molecules that control the activities of enzymes and other proteins. Work by Investigator John H. Exton, M.D., Ph.D. (Vanderbilt University) and his colleagues is directed toward identifying and chemically characterizing the enzymes that break down the lipids and the proteins that couple these enzymes to the receptors. Their work has led to the discovery of new mechanisms involving additional lipids, enzymes, and signaling molecules that are involved in the actions of the hormones. The enzymes and coupling proteins that participate in the new signaling systems are being characterized, and their physiological roles are being defined.

One emphasis of the research of Associate Investigator Linda J. Van Eldik, Ph.D. (Vanderbilt University) concerns the biological roles of the S100 family of calcium-modulated proteins. During the past year, her laboratory has focused on the S100β protein and its neurotrophic activity in the central ner-

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vous system. The major findings are that specific neurite extension activity and neuronal survival activity are two related activities inherent to the S100β molecule, that a disulfide-linked form of the protein is required for full biological activity, and that the relative position of the cysteine residues can be modified without loss of activity. These data suggest potential roles for S100β in the development and maintenance of neuronal function in the central nervous system and demonstrate the feasibility of longer term development of selective pharmacological agents based on the S100β structure.

The laboratory of Investigator Joan S. Brugge, Ph.D. (University of Pennsylvania) has continued investigating the importance of phosphorylation in regulating normal cellular events and in inducing oncogenic transformation. Structural studies on the c-*src* proto-oncogene product, a prototype protein tyrosine kinase, have identified a negative regulatory region outside of the catalytic domain. Mutational alterations in this region cause an elevation in the kinase activity of the *src* protein and activate its ability to alter the growth behaviors and morphology of cells. The c-*src* protein expressed in neurons contains an insertion of six amino acids within this negative regulatory region. Recent studies have indicated that this neuron-specific modification elevates the kinase activity of the protein. Investigations of the role of this protein in neurons are under way. The evidence that platelets express high levels of the c-*src* protein tyrosine kinase has led Dr. Brugge's laboratory also to examine the role of tyrosine phosphorylation in regulating the biological activity of platelets. Her studies suggest that tyrosine kinases may serve as second messengers in mediating events triggered by extracellular cell adhesion molecules.

The research interest of the laboratory of Investigator Charles T. Esmon, Ph.D. (Oklahoma Medical Research Foundation) is an understanding of the regulation of blood coagulation and the role of the clotting system in disease processes. The major approaches to these problems involve the protein C anticoagulant pathway and the prothrombin activation complex. Activated protein C, a vitamin K–dependent serine protease, expresses its anticoagulant activity in part through the inactivation of factor Va. Factor Va is a regulatory protein that binds to factor Xa, the vitamin K–dependent serine protease responsible for prothrombin activation, and accelerates the rate of prothrombin activation several thousandfold. Thus the interplay between the systems allows analysis of major regulatory events in coagulation. By selectively inhibiting specific components of these systems, Dr. Esmon can analyze the role of each pathway in selective disease processes in animal models.

Associate Investigator J. Evan Sadler, M.D., Ph.D. (Washington University) employs DNA cloning and biochemical methods to study blood coagulation. Many blood clotting proteins are made by the cells that line blood vessels or by white blood cells. These cells control such proteins to maintain a balance between promoting and preventing thrombosis. This balance is disrupted during inflammatory diseases that are associated with blood clots, such as bacterial infections. In the past year, three mechanisms were identified that regulate some of these proteins, and the structures of the genes for two of them were determined. One of these was the von Willebrand factor gene. Deletions in this gene were characterized in three patients with von Willebrand disease, the most common inherited bleeding disorder of humans.

The laboratory of Assistant Investigator Shaun R. Coughlin, M.D., Ph.D. (University of California at San Francisco) is pursuing two areas of importance in cardiovascular disease. 1) Mechanisms of thrombin action: thrombin is the most potent known activator of platelets, the cells that form plugs in blood vessels during blood clotting. Thrombin activates platelets by interacting with a "receptor" on the platelet surface. The identity of this receptor and the way it is activated by thrombin are unknown. Dr. Coughlin's laboratory has developed a number of novel approaches to identify and clone the thrombin receptor and to determine the mechanisms by which it is activated by thrombin. 2) The accelerated atherosclerosis of transplants. A rapid narrowing of the coronary arteries of transplanted hearts is a major cause of transplant failure. On the premise that accelerated transplant atherosclerosis represents an interaction of the recipient's immune system with the growth factor systems governing the proliferation of cells in arterial walls, Dr. Coughlin's laboratory, in collaboration with others, is identifying the immune cell types, growth factors, and growth factor receptors that are present during the development of transplant atherosclerosis in a rat model.

Investigator H. Franklin Bunn, M.D. (Brigham and Women's Hospital) and his colleagues have been investigating the gene for the hormone erythropoietin, which stimulates the formation of red blood cells. They have shown that exposure of erythropoietin-producing cells to low oxygen ten-

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sion increases both synthesis of the gene's messenger RNA and its stability. They have also prepared mutant erythropoietins and sequenced mammalian erythropoietins to obtain a better understanding of how the hormone acts. Finally, they have developed an accurate method for measuring small amounts of RNA and DNA. In addition, Associate James M. Cunningham, M.D., and his colleagues have identified a gene that encodes for a receptor protein that permits a leukemogenic murine retrovirus to infect mouse cells.

Mutations resulting from broken and rearranged chromosomes frequently produce an unusual mosaic phenotype in which the affected gene or genes are expressed in some cells but not others within a tissue. This year the studies of Investigator Allan Spradling, Ph.D. (The Carnegie Institution of Washington) on a small mutant chromosome, Dp1187, constructed from the fruit fly *Drosophila*, revealed that this phenomenon (position-effect variegation) is associated with drastic changes in chromosome replication near the site of breakage. To understand the molecular mechanisms involved, Dr. Spradling and his colleagues have localized several elements that regulate the replication of a specific chromosome region encoding *Drosophila* eggshell proteins. They have also initiated genetic studies of early steps in oogenesis, where chromosomes undergo fascinating processes of replication and recombination during meiosis.

Assistant Investigator Jeffrey Bonadio, M.D. (University of Michigan) and his colleagues have characterized an example of a class of collagen mutation that may be more common in the general population than previously suspected. Such mutations may be associated with relatively mild manifestations of connective tissue dysfunction. They have characterized also a mouse model of a form of human inherited connective tissue disease, osteogenesis imperfecta. The model provides an opportunity to investigate the effect of a reduced amount of type I collagen on the structure and integrity of extracellular matrix. This approach may provide a system in which therapeutic strategies to strengthen connective tissue can be developed.

Assistant Investigator James M. Wilson, M.D., Ph.D. (University of Michigan) and his colleagues have been developing new approaches to the treatment of metabolic diseases based on the transfer of genes into somatic cells. A major effort has been directed toward gene transfer into hepatocytes for treatment of inherited diseases associated with markedly elevated serum cholesterol levels. A rabbit animal model for an inherited form of hypercholesterolemia was used to develop and test several new genetic therapies. Expression of a recombinant gene that encodes a receptor capable of degrading low-density lipoproteins in hepatocytes of hyperlipidemic rabbits led to substantial decreases in total serum cholesterol. Dr. Wilson has also introduced recombinant genes into vascular endothelial cells that are capable of repopulating prosthetic vascular grafts *in vivo*. This technology has potential applications to the treatment of vascular disease and the design of new drug delivery systems.

The laboratory of Associate Investigator Gary K. Schoolnik, M.D. (Stanford University) is engaged in studies designed to determine how infectious agents cause disease. Microbial molecules that mediate the infectivity and virulence of an organism are identified, purified, and chemically characterized, and their corresponding genes are cloned. Immunologically interesting regions of these molecules are identified, and their efficacy as candidate vaccines is studied in animal models of human infectious syndromes. Those vaccines that exhibit protective efficacy are then assessed for their capacity to stimulate a safe immune response in human volunteers. Trials involving many human subjects are then conducted to assess the clinical utility of the vaccine. In this manner the laboratory seeks to prepare a new generation of safe and effective chemically defined vaccines. Specific organisms under investigation include *Neisseria gonorrhoeae*, *Moraxella bovis*, and *Yersinia enterocolitica*.

Associate Investigator Gerald R. Crabtree, M.D. (Stanford University) and his colleagues are studying the way in which cells of the immune system (T lymphocytes) develop the ability to carry out the complex functions required to defend the body from infection. The T lymphocytes circulating in the blood do not have the ability to perform immune functions until they come into contact with foreign antigens carried on invading organisms or transplanted tissues. This contact programs the T cell to develop an ability to destroy immunologically the cells carrying the foreign antigen. Work in Dr. Crabtree's laboratory has led to the identification of molecules that are likely to play an essential role in programming the T lymphocyte for immune function.

Cachectin or tumor necrosis factor (TNF) is a product of macrophages, released after exposure to a variety of infectious agents. It is of medical importance, since it acts to induce the state of shock that

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characterizes severe infections and the state of inanition (cachexia) that is observed in cancer and other chronic illnesses. Assistant Investigator Bruce A. Beutler, M.D. (University of Texas Southwestern Medical Center at Dallas) and his co-workers are studying the regulation of cachectin/TNF biosynthesis, the processing of cachectin/TNF once it has been produced, and the mechanism of macrophage activation by bacterial endotoxin.

The regulation of bacterial genes required for synthesis of the amino acid cysteine has been found to require a specific regulatory protein, CysB, which binds to the DNA of these genes. Acetylserine, which serves as a signal of cysteine deprivation, interacts with bound CysB protein and stimulates messenger RNA synthesis. In studies on mammalian sulfur metabolism, the laboratory of Investigator Nicholas M. Kredich, M.D. (Duke University) has cloned and sequenced DNA encoding the enzyme *S*-adenosylmethionine synthetase from rat liver. The product of the enzyme, *S*-adenosylmethionine, is required for more than 100 different cellular reactions and is a key factor in DNA, RNA, and protein metabolism.

CHARACTERIZATION OF TWO INSULIN-SENSITIVE DNA-BINDING PROTEINS

MARIA C. ALEXANDER-BRIDGES, M.D., PH.D., *Assistant Investigator*

The initial interaction of insulin with its cell surface receptor alters flux through diverse metabolic pathways culminating in alterations in cell growth, differentiation, and energy storage. The ability of insulin to stimulate glucose storage in a tissue-specific manner distinguishes this hormone from other anabolic peptide hormones and growth factors that alter growth-related processes. To describe fully the molecular mechanism of insulin's action on gene expression, Dr. Alexander-Bridges has been particularly interested in defining markers of insulin's metabolic effects, as well as markers of its growth-related effects on gene transcription. One gene encodes the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH); the other, the early growth response gene (*EGR1*), encodes a zinc finger protein. Using these markers, Dr. Alexander-Bridges has made considerable progress toward the goal of elucidating the molecular mechanisms that underlie the tissue-specific inductive effect of insulin on expression of certain metabolic enzymes. The regulation of these two insulin-sensitive genes has been extensively characterized *in vivo* in animal models of diabetes, with nutritional manipulations such as fasting and refeeding, and in tissue culture during differentiation of fibroblasts to adipocytes.

In ongoing studies aimed at elucidating the mechanism of insulin action on the expression of enzymes involved in glucose utilization, Dr. Alexander-Bridges has identified a target gene, GAPDH, that is regulated by insulin in adipose tissue and liver. GAPDH mRNA levels are induced 10-fold by insulin in cultured 3T3 adipocytes and in the epididymal fat pads of rats fasted and refed a high-carbohydrate, low-fat diet. Furthermore, expression of this gene is decreased to 30% of control levels in primary adipocytes isolated from diabetic animals and is increased threefold over control levels upon replacement of insulin. Because of the fidelity with which insulin regulation of GAPDH gene expression reflects the metabolic effects of insulin *in vivo*, subsequent efforts were focused on uncovering the molecular mechanisms of insulin action on this gene.

Because GAPDH mRNA levels are markedly increased by insulin in 3T3 adipocytes but not in 3T3 preadipocytes, Dr. Alexander-Bridges was interested in determining the mechanism of this tissue-specific response to insulin. Since the effect was a consequence of changes in gene transcription, she asked whether tissue specificity of the insulin response reflects the induction of a unique complement of transcription factors that are activated by acute exposure of 3T3 adipocytes to insulin.

To eliminate the possibility that acquisition of insulin-responsive gene expression during differentiation of 3T3 preadipocytes was due entirely to the presence of more insulin receptors on mature adipocytes, Dr. Alexander-Bridges examined the regulation of growth-related genes in these cells, thinking that the growth effects of insulin on gene expression in 3T3 fibroblasts might be detectable when metabolic effects of insulin were not. Dr. Perry J. Blackshear (HHMI, Duke University Medical Center) has shown that the proto-oncogene c-*fos* is regulated by insulin in 3T3 adipocytes through the well-described serum regulatory element (SRE), a factor that is present in fibroblasts. Although insulin can stimulate c-*fos* gene expression in 3T3 fibroblasts, the effects are small. The effect of insulin on *EGR1*, another serum-regulated gene, was very clear in 3T3 preadipocytes, however, and has provided a good marker for these studies. The fact that insulin could stimulate *EGR1* gene expression in preadipocytes indicated that the signal transduction pathway involved in mediating the growth response of insulin was intact and eliminated the trivial possibility that there were too few insulin receptors on the 3T3 preadipocyte to initiate an insulin response.

Dr. Alexander-Bridges then proceeded to determine what components of the signal transduction pathway of insulin's action on metabolic genes might be missing from 3T3 adipocytes. The approach was to work backward from an end effect of insulin on gene transcription by defining the factors that mediated the effect and determining whether they were expressed in a tissue-specific manner. In stably transfected cell lines the expression of a fusion gene containing the 5′-flanking sequences and promoter of the human GAPDH gene and the chloramphenicol acetyltransferase gene (HGAPDH-CAT) was stimulated threefold by insulin in 3T3 adipocytes and five- to eightfold in H35 hepatoma cell lines. Thus it appeared reasonable to assume that tissue-specific regulation of GAPDH was mediated at the transcriptional level. Analysis of Bal 31 deletion mutants of the HGAPDH-CAT construct indicated that the five- to eightfold stimulation by insulin was due to two independent se-

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quences, referred to as the upstream insulin regulatory element A (IRE-A) and downstream (IRE-B) elements, respectively. Both elements independently conferred a twofold inductive effect of insulin on gene expression and together resulted in a threefold effect.

The gel shift assay was used to detect transacting factors that interact with these elements. Both IRE-A and IRE-B interacted with insulin-sensitive DNA-binding proteins. Within 60 min of exposure of 3T3 adipocytes or H35 hepatoma cells to insulin, the activity of these sequence-specific DNA-binding proteins is increased at least fourfold. This effect is not inhibited by protein synthesis inhibitors. The IRE-A DNA-binding protein is induced 10-fold in liver and fat during the process of fasting and refeeding rats a high-carbohydrate, low-fat diet, again supporting the importance of GAPDH gene regulation *in vivo*. Finally, diabetic animals show a decrease in IRE-A DNA-binding activity and a marked stimulation of binding activity in extracts derived from insulin-treated diabetic animals.

To examine whether the acquisition of hormone-responsive GAPDH gene expression during differentiation of 3T3 preadipocytes to adipocytes was correlated with a change in activity of a DNA-binding protein, the laboratory has used the gel mobility shift assay to detect the interaction of nuclear proteins and IRE-A or IRE-B DNA. DNA binding correlated with the pattern of hormone-regulated GAPDH gene expression *in vivo* and in cultured cell lines. The IRE-A DNA-binding protein was barely detectable in 3T3 preadipocytes and was induced markedly by differentiation. The IRE-B DNA-binding protein was not detectable in 3T3 preadipocytes under any conditions but was highly responsive to insulin in 3T3 adipocytes. At the same time, the SRE-binding domain, which appears to mediate the effect of insulin on c-*fos* and EGR gene transcription, was used to show that equivalent amounts of SRE-binding activity had been extracted from 3T3 preadipocytes and adipocytes. These results indicated that the factors that mediate the growth-related effects of insulin on gene expression in 3T3 cells were constitutively present, while the factors that mediate the effect of insulin on GAPDH gene expression are expressed in differentiated tissues with lipogenic capacity. Thus the mechanism by which insulin achieves tissue-specific regulation of the GAPDH gene involves the induction of a novel set of transcription factors in terminally differentiated cells.

The activity of the IRE-A and IRE-B DNA-binding proteins is increased acutely and chronically by insulin. Efforts to understand the mechanisms that underlie this effect will be greatly facilitated by cloning these factors. Dr. Alexander-Bridges and her colleagues have isolated a clone that expresses a fusion protein capable of specific interaction with the IRE-A. Mutation of bases critical to the DNA-protein interaction inhibits binding of the fusion protein with IRE-A DNA. Further characterization of this clone is under way. Should further studies support the identity of this fusion protein as a transcription factor, antibodies to this protein will be raised and used to study the acute regulation of its DNA-binding activity in insulin-sensitive cells. Together these antibodies and cDNA clones will be used to examine the tissue distribution of this factor and to dissect the mechanism by which its expression and/or regulation are limited to specific tissues. Ultimately these studies will lead to an understanding of the mechanism by which insulin modulates the expression of specific genes involved in the maintenance of normal glucose and lipid metabolism. For example, GAPDH gene expression is markedly increased in the fat pads of obese Zucker rats but not in other insulin-sensitive tissues. With these clones, the regulation of IRE-A gene expression in the tissues of these animals can be examined. Understanding the hormonal control of lipid metabolism at a molecular level will provide invaluable insights into the mechanisms of obesity, a disease of fundamental importance in promoting diabetes.

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

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MECHANISM OF INSULIN ACTION

JOSEPH AVRUCH, M.D., *Investigator*

The overall goals of Dr. Avruch's work are 1) to identify the biochemical steps by which the insulin receptor tyrosine reorients cell function and 2) to develop methods to determine, in intact cells, the function of specific tyrosine and serine/threonine protein kinases.

I. Targets of the Insulin Receptor Tyrosine Protein Kinases.

An array of independently generated, high-affinity polyclonal and monoclonal antibodies against phosphotyrosine detect a common set of insulin-stimulated tyrosine P-containing polypeptides in a variety of cells. The role of these endogenous receptor kinase substrates as signaling intermediates in insulin's biologic actions remains invalidated. Several such proteins have been purified in the last year to essential homogeneity, and an analysis of the primary structure has been initiated, in an effort to establish the functional identity of these polypeptides. The major candidates are 180 kDa polypeptides and a doublet of ~65 kDa.

II. Insulin/Growth Factor–Stimulated Serine/Threonine Protein Kinases.

Earlier work from Dr. Avruch's laboratory had shown that one of the earliest responses to insulin was a stimulation of serine/threonine-specific phosphorylation of a subset of intracellular proteins. The view rapidly emerged that this phenomenon reflected the activation of one or more serine/threonine-specific protein kinases, as an intermediate signaling reaction consequent to receptor activation. The first insulin-stimulated serine/threonine phosphoprotein identified was the lipogenic ATP-citrate lyase. Subsequent work identified such other targets as 40 S ribosomal protein S6, acetyl-CoA carboxylase, and the insulin receptor itself. Recently an S6 protein kinase from livers of cycloheximide-treated rats was purified. Hepatic S6 phosphorylation increases markedly after cycloheximide administration *in vivo*. Concomitant with this is an increase in the activity of a protein kinase that is very active on S6; the chromatographic properties of this protein kinase are indistinguishable from the S6 kinase activated in regenerating rat liver or insulin-treated hepatoma cells. After purification the enzyme is visualized as a 70 kDa polypeptide that phosphorylates, in addition to S6, glycogen synthase, a number of nonhistone nuclear proteins, and ATP-citrate lyase; the last substrate is phosphorylated at sites identical to those phosphorylated in response to insulin *in vivo*. The activity of the purified S6 kinase is abolished by treatment with the serine/threonine phosphatase-2A.

Antisera raised to the 70 kDa S6 kinase polypeptide immunoprecipitate an insulin-stimulated S6 protein kinase activity from insulin-treated hepatoma cells. When hepatoma cells are labeled with ^{32}P, these immunoprecipitates exhibit a 70 kDa ^{32}P-peptide; the immunoprecipitation of ^{32}P-peptide is specifically inhibited by preincubation of the antisera with the purified S6 kinase. The ^{32}P content of this 70 kDa S6 kinase is increased approximately threefold by insulin treatment of the cells.

The 70 kDa rat liver S6 kinase is not cross-reactive with antisera raised to S6 kinase II purified from *Xenopus* oocytes or to a related recombinant *Xenopus* S6 kinase. These latter antisera do immunoprecipitate a distinct 85 kDa ^{32}P-polypeptide from rat liver, which also demonstrates S6 kinase activity in the immunoprecipitate. This 85 kDa ^{32}P-polypeptide is the rat liver homologue of *Xenopus* S6 kinase II. Low-stringency hybridization of rat liver cDNA libraries with the *Xenopus* cDNA has been used to obtain several cDNAs corresponding to this 85 kDa rat liver S6 kinase. Comparison of the deduced amino acid sequence for the rat liver 85 kDa S6 kinase to the sequence of peptide segments obtained from the purified 70 kDa S6 kinase reveals regions of considerable identity (~50%) within the protein kinase homology domain.

In summary, there appears to be a family of structurally and immunochemically distinct S6 protein kinases that is regulated by insulin and growth factors via phosphorylation on serine/threonine residues. The interrelations of these enzymes, the identity of the important substrates, and the protein kinases responsible for their activation are unknown.

Dr. Avruch and his colleagues have purified a second insulin-stimulated serine/threonine-specific protein kinase that phosphorylates microtubule-associated protein-2 (MAP-2). Thus activity of this enzyme is also abolished by serine/threonine dephosphorylation. The native substrates for MAP-2 kinase

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are unknown; MAP-2 kinase does not phosphorylate or reactivate the dephosphorylated 70 kDa hepatic S6 kinase.

Dr. Avruch is Professor of Medicine at Harvard Medical School and Physician at Massachusetts General Hospital.

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MEMBRANE-CYTOSKELETON INTERACTIONS

VANN BENNETT, M.D., PH.D., *Investigator*

The long-term goals of this laboratory are to advance understanding of the structural basis for long-range order in plasma membranes of mammalian cells. Research has focused on a system of proteins associated with plasma membranes of most animal cells, the spectrin-based membrane skeleton. Spectrin and its associated proteins are candidates for a role in localization of integral membrane proteins at specialized regions of the plasma membrane and in assembly of cell-cell junctions. As an initial approach to the functions of spectrin in complex tissues, this laboratory is examining the association of spectrin with the plasma membrane. Ankyrin is a spectrin-binding protein that links the spectrin skeleton to membrane proteins. Ankyrin-binding proteins include the anion exchanger of erythrocytes and cells of kidney collecting ducts, the $\alpha 1$ isoform of the Na^+,K^+ ATPase, and the voltage-dependent sodium channel of brain. These ankyrin-binding proteins have in common the feature of existing in specialized regions of the plasma membrane as an essential aspect of their function in tissues. The goal is to understand the molecular basis for the dual requirements of diversity and selectivity of protein interactions that allows ankyrin to associate with a number of different membrane proteins localized in distinct membrane domains.

I. Mechanisms of Ankyrin Diversity.

A. *Ankyrin isoforms.* The discovery of an association of ankyrin with the sodium channel in *in vitro* assays was unanticipated, since the sodium channel is highly localized to nodes of Ranvier and initial segments of axons, whereas ankyrin was believed to be distributed widely in brain. However, recent work indicates that ankyrin in brain and kidney is a family of closely related proteins that associate with spectrin but have different membrane binding sites and are targeted to distinct regions of the plasma membrane. Antibodies have been used to distinguish two variants of ankyrin in the nervous system. Localization of these ankyrin isoforms in brain has revealed that 1) one isoform is expressed only in neurons, whereas the other is present in both neurons and glial cells; and 2) the neuronal isoform of ankyrin is highly concentrated at nodes of Ranvier and initial axonal segments, sites that 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 nodes of Ranvier.

DNA encoding a human brain isoform of ankyrin has been isolated and partially sequenced. This isoform of brain ankyrin has extensive homology with human erythrocyte ankyrin in the spectrin-binding domain and in a region that associates with the anion exchanger and perhaps other membrane proteins. Brain ankyrin is the product of a distinct gene from erythrocyte ankyrin, and it is likely that additional genes will encode the ankyrin isoforms of kidney and other tissues. Another ankyrin isoform of human brain also has been cloned, although only limited sequence is available at this time. It will be important to determine the scope of the ankyrin family and to elucidate functional differences among family members. Dr. Bennett anticipates from previous work that different ankyrins will have distinct membrane receptors and associate with different isoforms of spectrin.

B. *Deletion of regulatory domains by alternative splicing of mRNA.* Erythrocytes contain a form of ankyrin missing a regulatory domain but retaining binding sites for spectrin and the anion exchanger. The shortened version of ankyrin exhibits enhanced binding to spectrin and to membrane sites. Dr. Bennett and his colleagues have discovered that the regulatory domain deleted from activated erythrocyte ankyrin is located internally within the protein sequence and thus results from alternative splicing of mRNA. Alternative splicing of mRNA leading to variation within the regulatory domain also is likely to occur in brain and provides an additional level of diversity within the ankyrin-family.

II. Structural Basis for Association of Erythrocyte Ankyrin with Membrane Proteins.

The binding sites involved in association between ankyrin and the anion exchanger have been explored. The ankyrin-binding site of the anion exchanger includes a region in the middle of the cytoplasmic domain but requires an extended sequence of at least 100 amino acids for full expression of activity. The anion exchanger–binding site of ankyrin has been localized to a 90,000-M_r amino-terminal fragment (90,000 domain) containing 22

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tandem, 33–amino acid repeats. Each repeat contains both highly conserved and variable areas of sequence. Ankyrin repeats are of particular interest, since very similar 33–amino acid repeats are found in proteins involved in developmental regulation in *Drosophila* and cell cycle regulation in yeast. Evidence was recently obtained that one or more of the repeats are likely to be involved in binding to the anion exchanger. It will be important to determine if the binding site involves variable or conserved regions within repeats, how many repeats are involved in binding, and whether repeats are utilized by other proteins that associate with ankyrin, such as the Na^+,K^+ ATPase and voltage-sensitive sodium channel. These studies will provide clues to the potential diversity of binding sites of ankyrin and evolution of ankyrin-binding proteins.

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

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REGULATION OF CACHECTIN/TUMOR NECROSIS FACTOR BIOSYNTHESIS

BRUCE A. BEUTLER, M.D., *Assistant Investigator*

Dr. Beutler and his colleagues are studying the mechanisms by which biosynthesis of cachectin [tumor necrosis factor (TNF)] is controlled. This important hormone, which is a product of peripheral blood monocytes and tissue macrophages, is produced in response to invasive stimuli, notably bacterial endotoxin. When released in large quantities, it acts to mediate many of the untoward effects of endotoxin, including shock, tissue injury, and coagulopathy. When released over a long period of time, it eventuates a state in which anorexia and unabating protein and lipid catabolism lead to weight loss and death. Cachectin/TNF has also been implicated as a mediator of several inflammatory processes in humans and animals, including the acute phase of graft-versus-host disease and the vascular changes that are observed in cerebral malaria. Thus a clear understanding of cachectin/TNF regulation would be medically significant.

Transcription of the cachectin/TNF gene is accelerated threefold after activation of the macrophage by endotoxin. However, cachectin/TNF mRNA levels rise by 100-fold or more, and at least a 10,000-fold increase in synthesis may be observed at the protein level. Thus much of the regulation of cachectin/TNF biosynthesis is post-transcriptional. This is supported by the observation that mRNA encoding cachectin/TNF and other cytokines often exists in an untranslated form.

Recent work in Dr. Beutler's laboratory has indicated that regulation of cachectin/TNF biosynthesis is largely exercised at a translational level. Reporter constructs, in which a chloramphenicol acetyltransferase (CAT)-coding sequence is driven by a constitutively active promoter unresponsive to the effects of endotoxin, but followed by varying portions of the cachectin/TNF 3′-untranslated region (UTR), have revealed that endotoxin-responsive sequences reside in the 3′-UTR of cachectin/TNF mRNA. The most important element within the 3′-UTR is the UpA-rich sequence commonly observed in many short-lived cytokine and proto-oncogene mRNAs, which has elsewhere been shown to confer translational suppression. Endotoxin appears to cause "derepression" of translation, leading to a >200-fold increase in the rate of reporter protein synthesis. Dexamethasone, which inhibits cachectin/TNF biosynthesis, also strongly inhibits the derepressive effect of endotoxin.

When cachectin/TNF is produced, it is synthesized as a prohormone containing (in the case of the murine protein) 79 propeptide amino acids appended at the amino terminus. The propeptide portion of the molecule is more highly conserved among species than the mature cachectin/TNF. It has been suggested that the protein is initially anchored in the plasma membrane and that membrane-associated cachectin/TNF may exist as an active species. Dr. Beutler and his colleagues have examined the processing of murine cachectin/TNF and have determined that a fraction of the protein exists as a glycosylated product. Moreover, they have found that a major alternative cleavage site exists, wherein the prohormone is cleaved before Leu_{-10}, yielding a soluble but inactive product. This suggests that the addition of several residues to the amino terminus is not tolerated, at least in the case of the murine protein. The extended form of the hormone retains a relatively high affinity for the cachectin/TNF receptor. This indicates that it may be possible to design competitive antagonists of cachectin/TNF binding. Given the inactivity of the precursor forms of cachectin/TNF, it is also possible that the protein may be regulated by proteolytic cleavage. This possibility is under study.

Dr. Beutler has also been interested in determining the nature of the signal evoked by endotoxin. This signal, which leads to the biosynthesis of cachectin/TNF, is apparently inoperative in C3H/HeJ mice. These mice are genetically unresponsive to endotoxin and therefore tolerate the administration of endotoxin in doses that are fatal to sensitive animals. Using macrophages of a line derived from the C3H/HeJ mouse as an assay substrate and adding back proteins derived from endotoxin-activated normal macrophages by electroporation, Dr. Beutler and his co-workers have demonstrated that a cytoplasmic protein induced by endotoxin acts to trigger the biosynthesis of cachectin/TNF. Currently research is directed toward the isolation and characterization of this protein, which may be central to the activation process.

The chief cellular *in vivo* sources of cachectin/TNF have remained mysterious, since the hormone is rapidly cleared and degraded after release. A nonsecreted marker protein, produced under circumstances identical to those evoking the release of cachectin/TNF, would help to clarify this issue. Using a marker construct in which the cachec-

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tin/TNF coding sequence has been precisely replaced by a CAT-coding sequence (with preservation of the promoter and downstream controlling sequences), Dr. Beutler and his associates will attempt to determine the site of cachectin/TNF production in a variety of disease models.

The studies currently in progress may illuminate the conditions required for macrophage activation by endotoxin and the molecular details involved in cachectin/TNF biosynthesis. They may also suggest practical means of interrupting these processes for therapeutic effect.

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

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INSULIN AND POLYPEPTIDE HORMONE ACTION

PERRY J. BLACKSHEAR, M.D., D.PHIL., *Investigator*

The mechanisms of action of insulin and related polypeptide hormones and growth factors are the major interest of Dr. Blackshear's laboratory. The laboratory is investigating the interactions among the often multiple intracellular reaction pathways activated after a given agonist of this type binds to its cell surface receptor. The activation of cellular processes by insulin is of primary concern, not only because the molecular mechanism of insulin action is clinically relevant but also because there is evidence that the tyrosine kinase pathways activated by insulin serve as a paradigm for similar pathways activated by certain growth factors.

I. Insulin Regulation of Protein Biosynthesis.

A. *Induction of* c-fos *proto-oncogene transcription by insulin and related growth factors.* Recent studies in Dr. Blackshear's laboratory have focused on the molecular mechanisms that are responsible for the induction of c-*fos* proto-oncogene transcription. In sensitive cells this strong response occurs within minutes of exposure to insulin, occurs with a dose-response relationship that parallels insulin receptor occupancy, and can be mimicked by a protein kinase C–independent pathway activated by various growth factors. In the past year, Dr. Deborah J. Stumpo has demonstrated that the protein tyrosine kinase activity of the insulin receptor is necessary for the insulin-stimulated effect to occur. The goal of these experiments is to link activation of this kinase to the rapid transcription of the c-*fos* gene by a series of biochemical reactions. Dr. Blackshear and his colleagues recently identified an element in the c-*fos* promoter that is responsible for the induction by insulin. Furthermore, Dr. Michael W. Roe has identified at least three proteins from cellular nuclear extracts that bind to this region of the gene with appropriate sequence specificity. Insulin stimulation of the cells results in an increase in the binding of one of these proteins to the specific promoter sequence of interest; this suggests that insulin is modifying the protein to cause this apparent increase in binding. Dr. Blackshear's laboratory is currently attempting to elucidate how this protein is modified in response to insulin treatment of cells; the answers to this question could lead to the activated insulin receptor protein kinase.

B. *Insulin-stimulated mRNA translation.* When sensitive cells are treated with insulin, numerous anabolic processes are activated, including generalized increases in protein synthesis and inhibition of protein breakdown. Dr. Richard M. Levenson has recently shown that part of this generalized increase in protein synthesis could be due to effects of insulin on a crucial component of the protein biosynthetic apparatus, elongation factor 2 (EF-2). Insulin not only rapidly stimulates the translation of EF-2 mRNA, it also appears to alter its phosphorylation state in such a way as to activate its function in stimulating nascent protein elongation. Dr. Levenson also found that, over and above a generalized effect of insulin on protein synthesis, approximately 35 proteins are synthesized much more rapidly in the presence of insulin, in most cases apparently by stimulation of mRNA translation. Joyce M. Manzella is concentrating on the mechanism of this translational activation by insulin, using the enzyme ornithine decarboxylase (ODC) as a model system. In recent studies involving the ODC mRNA 5′-untranslated region sandwiched between an exogenous promoter and a reporter gene, she has found that the ODC sequences dramatically inhibit translation of the reporter mRNA, both *in vitro* and *in vivo*. She has also determined that insulin can stimulate the translation of these mRNAs by a mechanism that involves the ODC sequences. She is currently attempting to determine which component of the ODC sequence is involved in both the constitutive inhibitory and insulin-stimulatory effects.

II. Molecular Characterization of a Prominent Protein Kinase C Substrate Protein.

Within the past year, Dr. Stumpo and Dr. Jonathan M. Graff have cloned, sequenced, and expressed cDNAs encoding a major cellular substrate for protein kinase C, which they have named the myristoylated alanine-rich C kinase substrate (MARCKS). They have determined that two regions of sequence identity between the chicken and bovine proteins are involved in three different behaviors of the proteins: myristoylation, phosphorylation, and calmodulin binding. Dr. Graff determined that a mutation that prevented amino-terminal myristoylation of the protein caused it to remain exclusively in the cytosol, yet the protein remained

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an excellent substrate for protein kinase C in intact cells, in contrast to the current view that substrates for this kinase need to be membrane-associated. He also found that the protein contained four serines phosphorylated by protein kinase C that were clustered in a 25–amino acid basic domain, the phosphorylation site domain. Synthetic peptides comprising this domain were phosphorylated by protein kinase C with high affinity and positive cooperativity; a peptide in which all four serines were replaced by alanines behaved as a potent pseudosubstrate inhibitor for the kinase.

Dr. Graff also found that the MARCKS protein could bind calmodulin and that this binding was prevented by prior phosphorylation of the protein by protein kinase C. This led to the finding that the phosphorylation site domain and the calmodulin-binding domain were contiguous or identical, a prediction confirmed by the finding that the phosphorylation site domain peptide could bind calmodulin with high affinity. The current hypothesis is that calmodulin and the MARCKS protein exist in cells as a complex under conditions of protein kinase C inactivity: when the kinase is activated (almost always in situations in which cellular calcium concentrations are transiently elevated) the components of the complex separate; the "free" calmodulin could then act, in concert with the elevated calcium levels, to activate various calmodulin-activated enzymes. This might provide a mechanism for the synergistic interaction between the two pathways that has been noted.

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

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PROTEIN TRAFFIC ACROSS MEMBRANES

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

Research in Dr. Blobel's laboratory is focused on four protein translocation systems (translocons) that function in the translocation of cytoplasmically synthesized proteins across (or integration into) 1) the endoplasmic reticulum (ER), 2) the prokaryotic plasma membrane, 3) the chloroplast envelope, and 4) the two mitochondrial membranes.

Each of the four translocons is likely to consist of at least four entities. 1) A cytosolic signal-recognition factor that recognizes and binds to a signal sequence, thereby segregating the signal sequence from the rest of the chain and preventing inactivation of the signal sequence (inactivation might occur rapidly if the signal sequence were free to fold with the rest of the chain and be no longer accessible on the surface of the folded preprotein). 2) A "homing" receptor that is part of the membrane-bound components of a given translocon and functions as the cognate receptor for a given signal-recognition factor. After binding of signal-recognition factor to its homing receptor, the signal-recognition factor is dissociated from the signal sequence. These steps target the signal sequence to the membrane and permit it to interact with a second membrane-bound signal-recognition system, which is part of a protein-conducting channel. 3) A ligand-gated protein-conducting channel that would possess a cytosol-exposed signal sequence–binding domain and that would open in response to ligand binding, i.e., binding of the signal sequence to this domain. The channel would close after completion of translocation and open again only after binding of another signal sequence. This channel (unlike ligand-gated ion channels) could also open in a second dimension, namely to the lipid bilayer, as a result of encountering a stop-transfer sequence. In this case the portion of the chain that is located in the channel would be extruded, through this opening, from the channel to the lipid bilayer, followed by closing of the channel, in both dimensions. As a result a portion of the chain would be integrated into the lipid bilayer. Accessory proteins could function on either side of the channel, perhaps as protein antifolding factors. 4) A signal peptidase removes the signal peptide, in many cases. This enzyme is part of the trans components of the translocon (the cis components are the cytosolic or cytosol-exposed components).

Data were published from this laboratory in the past year on all four translocons.

I. Endoplasmic Reticulum Translocons.

A. *ER translocon of canine pancreas.* By fusing rough microsomes with plasma lipid bilayers, Dr. Sanford Simon, in collaboration with Dr. Joshua Zimmerberg (National Institutes of Health) has detected large aqueous channels in the ER membrane. These channels were mostly open at negative membrane potential at the cytoplasmic site of the membrane and closed at positive voltages. There was a dramatic increase in the number of open channels when 100 μM GTP was added, whereas 100 μM GTPγS caused closing of channels. A similar channel was also detected when inverted vesicles derived from the plasma membrane of *Escherichia coli* were fused. Because both membranes share the ability to translocate secretory proteins, it is likely that this channel is the long-sought-after protein-conducting channel.

In 1986, signal peptidase was isolated in Dr. Blobel's laboratory as a complex of at least five polypeptide chains. Drs. Gregory Shelness and Yashpal Kanwar have sequenced the cDNA for the glycoprotein subunit of the complex. The deduced amino acid sequence shows one classical transmembrane segment and one glycosylation site. It is not similar to any other protein in the data bank.

Dr. Christopher Nicchitta has used chemical alkylation to distinguish the process of nascent chain targeting and signal sequence insertion from subsequent chain translocation across the membrane. Translocation across the membrane is mediated by an *N*-ethylmaleimide–sensitive membrane protein. This protein might be part of the protein-conducting channel.

A trimeric G protein of the α-, β-, γ-subunit type was detected in the ER by Drs. Yves Audigier and Sanjay Nigam. The function of this G protein, which does not appear to be involved in protein translocation, remains to be investigated.

B. *ER translocon of yeast.* In 1986 Dr. Blobel's laboratory, and independently two other laboratories, developed yeast cell-free systems that faithfully reproduce protein translocation across yeast microsomes. Components of the yeast ER translocon have not been isolated. However, Drs. William Chirico and Gerald Waters succeeded in isolating two closely related 70 kDa heat-shock proteins (98% homologous) of yeast cytosol that are re-

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quired for post-translational protein translocation in yeast. This unexpected result has made a major impact on assigning functions to these proteins. In addition, Drs. Chirico and Water have shown that another *N*-ethylmaleimide-sensitive factor is required. It is likely that this factor represents a signal-recognition factor.

Dr. Jacques YaDeau has succeeded in solubilizing yeast microsomal signal peptidase and developed an *in vitro* assay for measuring its activity. This work should pave the way for the isolation of the enzyme.

The substrate that was used in Dr. YaDeau's work was yeast prepro-α factor. Drs. Gerald Waters and Emily Evans have shown that this protein has a cleavable signal sequence.

II. Prokaryotic Plasma Membrane Translocon.

Previously this laboratory showed that a soluble factor (presumed to be a signal-recognition factor) was required for protein translocation across the *E. coli* plasma membrane. Dr. Makoto Watanabe has now purified this factor to homogeneity. This protein is a 64 kDa homotetramer consisting of four identical 16 kDa subunits. Amino-terminal sequence analysis revealed that the 16 kDa protein is identical to the *secB* gene product. Dr. Watanabe has shown that the *secB* tetramer functions as a signal-recognition factor that binds to the signal sequence of preproteins and can compete with the signal-recognition particle of the ER translocon for binding to the signal sequence.

Dr. Watanabe also has obtained biochemical evidence that PrlA (SecY) of *E. coli* functions as a membrane-bound signal-recognition system. Fab fragments of antibodies raised against synthetic peptides representing the amino- and carboxyl-terminal domains of this protein inhibited translocation by interfering with the binding of the preproteins to the membrane.

III. Chloroplast Envelope Translocon.

Using an antiidiotypic antibody approach, Drs. Debkumar Pain and Yashpal Kanwar have identified the first component of the chloroplast envelope translocon. Antibodies were raised in rabbits against a synthetic signal peptide. An IgG fraction of the anti–signal peptide antibodies was then used to raise antiidiotypic antibodies that mimic the signal peptide. Fab fragments of these antibodies inhibited protein import into chloroplasts. Polypeptides of the chloroplast envelope resolved by SDS-PAGE, transferred to nitrocellulose, and then probed with the antiidiotypic antibodies reacted with a 30 kDa integral membrane protein. Immunofluorescence of isolated chloroplasts yielded punctate staining, and immunoelectron microscopy showed decoration of the so-called contact zones between outer and inner chloroplast membrane. It is likely that this protein is a signal sequence–binding subunit of a protein-conducting channel in the outer chloroplast membrane that is linked to a similar channel in the inner chloroplast membrane, thus giving rise to the morphological phenotype of contact zones.

IV. Mitochondrial Membrane Translocon.

Except for signal peptidase, none of the components of the mitochondrial membrane translocon have been isolated. Drs. Hiroshi Murakami and Debkumar Pain have demonstrated that protein import into mitochondria requires hsp70 (see *ER translocon of yeast*) and an *N*-ethylmaleimide-sensitive cytosolic factor. The latter is presumed to be a signal-recognition factor.

V. Structure and Function of the Nuclear Lamina and Pore Complexes.

A. *Higher eukaryotes.* Drs. Howard Worman and Spyros Georgatos and graduate student Jeffrey Yuan have identified a 58 kDa lamin B receptor in the nuclear envelope of turkey erythrocytes. The protein is an integral membrane protein.

Drs. Richard Wozniak and Eckart Bartnik have cloned and sequenced the cDNA for an integral membrane glycoprotein (gp210) that is localized in the membrane limiting the nuclear pore. The deduced amino acid sequence revealed that gp210 has one classical transmembrane segment and another hydrophobic region that could function as a fusogenic peptide. It is possible that this protein functions in the formation of pores and in anchoring of the nuclear pore complex.

B. *Yeast.* Dr. John Aris has identified two nuclear envelope proteins in yeast that cross-react with antisera against mammalian nuclear pore complex proteins. Immunofluorescence and electron microscopy data suggest that these cross-reactive proteins are yeast nuclear pore complex proteins. Drs. Spyros Georgatos and Ionna Maroulakou have used various binding assays and cross-reactive anti-

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bodies raised against the turkey proteins to identify lamin A, lamin B, and lamin B receptor in *Saccharomyces cerevisiae*. The identification of these components in yeast should allow the use, in addition to biochemical approaches, of genetic approaches to investigate the function of these proteins.

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

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STRUCTURE-FUNCTION RELATIONSHIPS IN TYPE I COLLAGEN

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

Dr. Bonadio's laboratory is interested in the relationship between structure and function for type I collagen, a fibrous protein that resides in the extracellular matrix of most tissues. Type I collagen was chosen partly because of a larger interest in the contribution of the matrix to tissue assembly during development. The goal is to define this contribution in molecular terms, by focusing on a major component of the matrix. The heteropolymeric nature of collagen, its abundance, its supramolecular assembly into fibrils, its structural role, and its organization into a connective tissue matrix distinguish collagen from many other proteins and imply that unique structure-function relationships exist.

I. Characterization of an Osteogenesis Imperfecta Mutation.

The opportunity to characterize naturally occurring mutations provides a powerful set of sequence alterations that, given their association with human disease, are assumed to occur within important domains in the molecule. The laboratory recently described a homozygous, G→A transition at the moderately conserved +5 position within the splice donor site of intron 14 in the human α1(I) collagen gene. The mutation reduced the efficiency of normal splice-site selection, since the exon upstream of the mutation was alternatively spliced. Moreover, the extent of alternative temperatures at which the mutant cells were grown suggested that the mutation had a direct effect on spliceosome assembly. The homozygous mutation was associated with a lethal phenotype. However, inefficient exon skipping suggests that splicing mutations in collagen genes may be more widespread in the general population than previously recognized. In other words, low-level expression of alternative splicing (e.g., with heterozygous mutations) may be associated with mild dysfunction of connective tissue.

II. Transgenic Mouse Model of the Mild Dominant Form of Osteogenesis Imperfecta.

Osteogenesis imperfecta type I (OI-I) is a nonlethal disorder characterized by bone fracture without deformity, blue sclerae (and other defects in nonmineralized connective tissue), normal or near-normal stature, and autosomal dominant inheritance. The incidence of OI-I is estimated to be 1/20,000 live births; males and females are affected equally. Osteopenia is associated with an increased rate of long-bone fracture upon ambulation. Presenile hearing loss is a feature of this disease in about half the families. All cases to date have been associated with mutations in the extracellular matrix molecule type I collagen. Most affected individuals are thought to have heterozygous, null collagen mutations, although no naturally occurring mutation of this type has been characterized to date at the molecular level.

The *Mov13* strain was generated by the laboratory of Dr. Rudolf Jaenisch by exposing mouse embryos to Moloney murine leukemia virus. Genetic and molecular evidence indicated that a single copy of the provirus integrated into the first intron of the α1(I) collagen gene. The proviral insert is associated with a change in chromatin conformation and *de novo* methylation of the gene, and it prevents initiation of transcription. Mice homozygous for the null mutation die *in utero* due to failure of the vascular system. However, heterozygous *Mov13* mice do not display an obvious mutant phenotype. Dr. Bonadio's group undertook a detailed biochemical and functional analysis to determine whether *Mov13* mice could serve as a model of OI-I.

A defect in type I collagen production was associated with dominant, morphological and functional defects in mineralized and nonmineralized connective tissue and with progressive hearing loss. Therefore the mutant mice represent a faithful model of OI-I. The model provides an opportunity to investigate the effect of a reduced amount of type I collagen on the structure and integrity of extracellular matrix. It also may represent a system in which therapeutic strategies to strengthen connective tissue can be developed.

III. Biomineralization.

The perinatal lethal form of osteogenesis imperfecta (OI-II) is characterized by short stature, a soft calvarium, blue sclerae, fragile skin, a small thoracic cage, and floppy-appearing lower extremities (due to external rotation and abduction of the femurs). Radiographic signs of bone weakness include compression of femurs, bowing of tibiae, broad and beaded-appearing ribs, and poor mineralization (of both membranous and endochondral bone). The reasons for bone deformation and poor

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mineralization are not clear. However, it has been suggested that the abnormal radiographic appearance results from an inability of bones deficient in organic matrix and mineral to resist skeletal muscle activity *in utero*.

The elastic modulus of bone is a composite of the material properties of its mineral and organic phases. An extracellular matrix defect (i.e., a defect within the organic phase of bone) primarily is responsible for skeletal fragility in OI-II patients. All OI-II cases to date have been associated with structural mutations in genes encoding the constituent α-chains of type I collagen, the most abundant protein in bone. These mutations alter the function of type I collagen by disrupting the conformation of molecules that incorporate mutant α-chains.

For some time it has been argued that a change in collagen conformation logically would affect mineralization. The collagen molecule exists in tissues as a fibril. The collagen fibril is a heteropolymer in which collagen molecules are packed in a highly ordered, staggered, three-dimensional array. A series of gaps between molecules are created that serve as sites of mineral (largely calcium hydroxyapatite) deposition. A change in collagen conformation could disrupt fibril architecture and interfere with biomineralization. Experimental evidence demonstrating abnormal fibrils within the OI-II extracellular matrix is noteworthy in this regard.

The laboratory has endeavored to establish a model system to study systematically the effects of OI-II mutations on biomineralization. A novel method to isolate, purify, and propagate d16 chick embryo calvarial cells in cell culture has been developed. The cultured cells elaborate and mineralize a type I collagen-based extracellular matrix. The cells are transiently transfectable at rates comparable to primary d16 chick embryo tendon cells. In addition, a full-length chick α1(I) cDNA has been isolated. The model system should allow an analysis of the relationship between collagen conformation, fibril formation, and mineral deposition by the expression of mutagenized cDNAs in osteoblast cells.

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

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REGULATION OF TYROSINE PHOSPHORYLATION

JOAN S. BRUGGE, PH.D., *Investigator*

Dr. Brugge's laboratory is examining the role of tyrosine phosphorylation in normal cells and the mechanism of oncogenic transformation by mutated variants of tyrosine kinases. As a model system for these studies, the cellular *src* protein, a 60 kDa protein tyrosine kinase, has been investigated.

I. Tyrosine Phosphorylation in Platelets.

Platelets contain very high levels of the protein tyrosine kinase $pp60^{c\text{-}src}$. This regulatory enzyme represents 0.2–0.4% of total cellular protein in these cells. To determine whether changes in tyrosine phosphorylation accompany events involved in platelet activation, Andy Golden and Dr. Brugge have examined the pattern of tyrosine phosphorylation after treatment with agents that activate platelet functions. Thrombin, a potent activator of platelet secretion and aggregation, caused rapid changes in tyrosine phosphorylation of multiple platelet proteins, predominantly a 95–97 kDa protein doublet. Other agonists, including collagen, mastoparan, calcium ionophores, and ADP plus fibrinogen also induced similar changes in tyrosine phosphorylation; however, forskolin, which blocks thrombin-induced secretion and aggregation (by increasing the levels of intracellular cAMP) prevented the induction of tyrosine phosphorylation by thrombin and other agonists. These results suggested that the activation of tyrosine phosphorylation was not specifically coupled to the thrombin receptor and that tyrosine phosphorylation may mediate some of the changes in cell physiology that are triggered by extracellular signals.

To investigate the mechanism responsible for activation of tyrosine phosphorylation in platelets, Drs. Brugge and Golden used a variety of agonists and inhibitors of specific platelet functions to establish whether phosphorylation of the 95–97 kDa protein doublet correlated with events associated with platelet aggregation (i.e., fibrinogen receptor occupancy) or with events that activate platelet secretion. Platelet aggregation was blocked with reagents that interfere with the interaction between fibrinogen and its integrin-like receptor, gpIIb–IIIa [monoclonal antibodies to the fibrinogen receptor (provided by Dr. S. Shattil, University of Pennsylvania) or the peptide RGDS, which occupies the fibrinogen-binding region of the receptor]. Platelet secretion was blocked using indomethacin in combination with the agonist ADP and fibrinogen. ADP induces fibrinogen binding to its receptor, leading to primary aggregation, which is followed by arachidonic acid metabolism and subsequent granule secretion. Indomethacin inhibits secretion by blocking the oxidation of arachidonic acid metabolites. The effectiveness of each of these reagents was monitored using either a lumi-aggregometer or a fluorescence-activated cell sorter (FACS) (in collaboration with Dr. Shattil) to monitor fibrinogen receptor exposure, occupancy, or granule fusion (secretion). Incubation with the peptide RGDS or antibodies to the fibrinogen receptor prevented platelet aggregation and blocked the phosphorylation of the 95–97 kDa protein doublet. Incubation with indomethacin in the presence of ADP and fibrinogen did not affect platelet aggregation but blocked platelet secretion. Under these conditions, phosphorylation of the 95–97 kDa doublet was not affected. These results indicate that tyrosine phosphorylation of the 95–97 kDa doublet does not require the process of secretion nor the products of secretion; however, this phosphorylation depends on events that are triggered by interaction between fibrinogen and its receptor. These results suggest that a tyrosine kinase(s) may be directly or indirectly coupled to the fibrinogen receptor in platelets. In addition, these findings also suggest that tyrosine kinases may serve as second messengers to relay signals that are transmitted by interaction between extracellular matrix proteins and/or cell adhesion molecules and their receptors.

II. Functional Activity of a Neuron-specific Variant of the c-*src* Protein.

Neurons express a structurally distinct form of the c-*src* gene product that possesses a higher specific activity than the c-*src* protein expressed in other cell types. Dr. Joan Levy cloned c-*src* cDNA species from a chicken brain library. These cDNAs contained an 18-nucleotide insertion at the junction between two exons, suggesting that the messenger RNA from neurons uses an alternate pattern of RNA splicing. Dr. Levy constructed a retroviral vector that allowed expression of this brain-specific c-*src* cDNA to compare the functional activity of this variant form of the c-*src* protein, $pp60^{c\text{-}src+}$, with the insert-minus form of this protein, $pp60^{c\text{-}src}$, expressed in other cell types. The $pp60^{c\text{-}src+}$ protein

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expressed in fibroblasts displayed two- to fourfold higher specific activity than insert-minus pp60$^{c\text{-}src}$. In addition, the c-*src*$^{+}$ protein expressed in fibroblasts was not phosphorylated at the serine residue previously shown to be phosphorylated in pp60$^{c\text{-}src+}$ synthesized in neurons. These results indicated that the six–amino acid insertion in pp60$^{c\text{-}src+}$ was sufficient to cause an increase in the specific activity of the c-*src* protein. Chicken embryo fibroblasts that expressed high levels of this protein displayed an altered morphology compared with cells that overexpressed pp60$^{c\text{-}src}$ (slightly more refractile and less contact-inhibited). In addition, the c-*src*($^{+}$) overexpressor cells formed larger colonies in soft agar than the insert-minus c-*src* overexpressors and displayed higher levels of tyrosine phosphorylation. Thus the six–amino acid insertion outside of the catalytic domain of pp60$^{c\text{-}src}$ modulates the kinase activity of this protein, causing an increase in its kinase activity *in vitro* and *in vivo*. Current studies are focused on localization of pp60$^{c\text{-}src+}$ in neural tissues and elucidation of the functional role of this protein in neurons.

III. Modulation of the Functional Activity of the c-*src* Protein through Site-directed Mutagenesis.

Several lines of evidence indicate that sequences within the amino-terminal half of pp60$^{c\text{-}src}$ serve to regulate the catalytic activity of this protein tyrosine kinase. To examine how mutations outside of the catalytic domain of *src* influence the functional activity of the molecule, Larry Fox, Susan Nemeth, and Mike DeMarco have constructed and analyzed mutant forms of the protein containing single–amino acid substitutions and deletions throughout the amino-terminal half of the molecule. Mutations within the A homology box (residues 88–138, which are shared with seven *src*-related kinases, γ-phospholipase C, brain α-spectrin, and the v-*crk* oncogene) caused an activation of the kinase activity and the transforming potential of the c-*src* protein. Cells expressing elevated levels of these mutant proteins possessed elevated levels of tyrosine phosphorylation, produced higher levels of plasminogen activator, formed larger colonies in soft agar, and showed a more refractile and transformed cell morphology. The degree of activation correlated with the extent of the mutation, with single–amino acid substitutions causing the most subtle activation, and deletions of up to 75 amino acids, the strongest activation. These results suggest that sequences within this region of pp60$^{c\text{-}src}$ interact with the catalytic domain to maintain the protein in a low-activity state. Alterations within this region appear to relax this interaction and cause an activation of c-*src* protein kinase activity. Mutations within the A box region have been combined with mutations in the B homology box and with mutations within the carboxyl-terminal half of *src*; synergistic, additive, or suppressive interactions between these domains have been examined. The analysis of these mutations is complicated; however, the following conclusions can be drawn: 1) Sequences within the B box [residues 148–190, which are shared with *src*-related tyrosine kinases, the GTPase-activating protein (GAP) of the *ras* protein, and γ-phospholipase C] are not necessary for maintenance of the high kinase activity of all activated variants of c-*src*. 2) Elimination of the B box caused a severe restriction in the phosphorylation of most substrates *in vivo* (without affecting *in vitro* kinase activity, or the phosphorylation of several substrates *in vivo*, e.g., p36, calpactin). 3) Phosphorylation of tyrosine 416 is necessary for full expression of the biological activity of all activated forms of c-*src*, yet is not necessary for expression of a high catalytic activity of all transforming variants of c-*src*. These studies strongly suggest that sequences within the B box and phosphorylation of tyrosine 416 affect the expression of the biological activity of the *src* protein in subtle ways that do not directly affect the catalytic activity *per se*. This work was also supported by grants from the National Cancer Institute.

Dr. Brugge is also Professor of Microbiology at the University of Pennsylvania School of Medicine.

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REGULATION OF ERYTHROPOIESIS

H. Franklin Bunn, M.D., *Investigator*

Dr. Bunn and his colleagues have been investigating the biogenesis and the mechanism of action of erythropoietin, the hormone that regulates the production of red blood cells in humans and other mammals. Dr. James Cunningham's research focuses on the murine ecotropic receptor for retroviruses.

I. Structure-Function Relationships of Erythropoietin.

Erythropoietin (Epo) is a highly soluble protein containing 166 amino acids and 40% carbohydrate. Because it has not yet been crystallized, there is no information on its three-dimensional structure. Preliminary computer-based modeling, done in collaboration with Dr. Fred Cohen (University of California at San Francisco) suggests that Epo is homologous to human growth hormone (20 kDa) and assumes a right-handed four-helix bundle structure, with antiparallel adjacent α-helices. Dr. Jean-Paul Boissel and Dr. Bunn created a set of deletion mutants that excised each of the potential helices. In all cases, *N*- and *O*-glycosylation sites were preserved. Human Epo cDNA and the mutated cDNAs were inserted into a mammalian vector and transiently expressed in COS7 cells. When compared with the normal Epo construct, nearly the same mRNA levels were observed for all the mutants, but no protein was detected in either the cells or the media by both radioimmunoassay and *in vitro* bioassay. These results suggest that these deletions resulted in very unstable translation products. In contrast, partial deletion of a predicted interconnecting nonhelical loop gave rise, after transient expression, to a protein produced and secreted nearly as effectively as the Epo wild type. This structural model predicts that this deletion should not destabilize the molecule. Moreover, this mutant exhibited full biological activity. Another approach to studying structure-function relationships is to generate and compare Epo sequences from a variety of mammals. A large set of primers corresponding to sequences conserved between mouse and human were synthesized in order to amplify by means of the polymerase chain reaction (PCR) genomic Epo fragments from cell lines of 10 mammalian species covering eight orders. Restriction enzyme mapping verified that these amplified fragments originated from the corresponding animals. Comparison of these sequences should provide insights into important functional domains in the Epo molecule.

II. Regulation of the Erythropoietin Gene.

The human hepatoma cell line Hep3B synthesizes large quantities of Epo in a regulated manner in response to hypoxia and cobaltous chloride ($CoCl_2$). This regulation occurs at the Epo mRNA level. To delineate further the nature of the regulation of Epo gene expression, Dr. Mark Goldberg and Dr. Bunn have studied the effects of hypoxia (1% O_2) and $CoCl_2$ on the rate of Epo gene transcription. Although Northern blot analyses showed that steady-state Epo mRNA levels increase more than 100-fold in response to hypoxia or $CoCl_2$, nuclear runoff experiments demonstrated only a 5- to 10-fold increase in Epo mRNA transcription in response to these stimuli. Experiments were therefore undertaken to investigate post-transcriptional influences on steady-state Epo mRNA levels. When Hep3B cells were grown in 1% O_2 to increase Epo mRNA levels, and then switched to 21% O_2, Epo mRNA had a rapid rate of decay, with steady-state levels falling by 50% within 1.5–2 h and with almost undetectable levels by 6 h. This finding is similar to reported changes in kidney Epo mRNA levels in mice switched from a hypoxic to a nonhypoxic environment. To distinguish between transcriptional and post-transcriptional contributions to this observed fall in steady-state Epo mRNA levels, actinomycin D chase experiments were performed. Hep3B cells were made hypoxic to allow an increase in Epo mRNA levels. After the addition of actinomycin D the cells were either kept hypoxic or allowed to equilibrate in 21% O_2 for various periods of time. The stability of the Epo mRNA in the presence of actinomycin D at both O_2 tensions was much greater than that observed in the absence of actinomycin D and much greater than that which has been reported *in vivo*. If actinomycin D was preventing the transcription of a rapidly turning over mRNA whose product destabilizes Epo mRNA, cycloheximide would be expected to have a similar effect on the stability of Epo mRNA. Subsequent experiments did demonstrate a reproducible increase in the stability of the Epo mRNA in the presence of cycloheximide. Thus it appears that the induction of Epo mRNA in response

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to hypoxia is determined by both increased transcription and a novel mechanism for increasing RNA stability.

III. Measurement of mRNA by Competitive Polymerase Chain Reaction.

Understanding the regulation of hematopoiesis depends in part on the ability to measure mRNA species of hematopoietic growth factors in defined cell populations accurately. Conventional methods of mRNA analyses are not sensitive enough to detect mRNA in samples limited by either low cell number or low copy number per cell and permit only crude quantitation of mRNA. Because of extraordinarily high sensitivity, PCR is being used for amplifying cDNA copies of low-abundance mRNA. However, quantitation has been unreliable, because the amount of PCR product increases exponentially with each cycle of amplification, and therefore minute differences in any of the variables that affect the rate of amplification can alter product yield dramatically. Drs. Gary Gilliland and Kerry Blanchard and Dr. Bunn have devised a simple and inexpensive method utilizing competitive PCR for highly accurate quantitation of mRNA from a small number of cells. Serial dilutions of a competitor DNA fragment that differs from the cDNA of interest only by having either a small intron or a mutated internal restriction site are added to aliquots of the PCR reaction mix. Therefore the same primers are used to coamplify the unknown and the competitor. The ratio of products remains precisely constant through the amplification. The relative amount of each provides a precise measure of cDNA concentration. This technique has been used to quantitate mRNA levels of three inducible cytokines: interleukin-3 (IL-3) and granulocyte macrophage colony-stimulating factor (GM-CSF) in MLA-144 cells before and after induction with phorbol myristate acetate (PMA) and Epo in Hep3B cells before and after induction with hypoxia. Messenger RNA was undetectable in unstimulated cells by PCR. However, with appropriate induction, mRNA for each of these cytokines was detected and quantitated from as few as 100 cells. This strategy can be applied to a variety of studies on gene expression requiring accurate measurement of mRNA species in low abundance or in small numbers of cells isolated by fluorescent sorting or plucked from colonies grown in semisolid medium. Moreover, competitive PCR should also be useful in analyses of DNA, such as measurement of gene dosage (both amplification and deletion) or quantitation of low-abundance DNA species, e.g., somatic cell mutations and integration of viral DNA, both of which may involve only a small minority of a cell population.

IV. Identification of a Gene Encoding a Retrovirus Receptor.

Murine type C ecotropic retrovirus infection is initiated by virus envelope binding to a membrane protein expressed on mouse cells. Dr. Cunningham and his colleagues have identified a cDNA clone that encodes for this protein. Human EJ cells that express the cDNA acquire a millionfold increase in virus infectivity. The predicted 622–amino acid sequence of this putative receptor is extremely hydrophobic; 14 potential membrane-spanning domains have been identified. A model of the structure of the protein in the membrane is different from other virus receptors, and a computer-based search of sequence data banks has not identified another protein with significant similarity. Therefore this protein may function in a novel way to permit virus entry into the cell. The structural basis for virus interaction with the receptor is currently being investigated by examination of chimeric receptors constructed by exchanging small regions of the receptor cDNA with its human homologue (which is nonpermissive for infection). These experiments have identified a single domain that is responsible for the virus receptor function. Site-directed mutations within this domain are being prepared to identify the specific amino acid residues required for virus infectivity.

Dr. Bunn is Professor of Medicine at Harvard Medical School and Director of Hematology Research at Brigham and Women's Hospital.

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HORMONAL REGULATION OF GENE EXPRESSION

WILLIAM W. CHIN, M.D., *Investigator*

Dr. Chin and his colleagues have focused on the molecular mechanisms involved in hormonal regulation of gene expression, emphasizing thyroid hormone regulation of thyrotropin [thyroid-stimulating hormone (TSH)] and sex steroid/gonadal peptide regulation of gonadotropin [lutropin (LH) and follitropin (FSH)] gene expression. In addition, Dr. Chin's studies on the molecular biology of thyroid hormone receptors (THRs) have underscored them as prototypes of trans-acting factors involved in the regulation of gene expression.

I. Thyroid Hormone Regulation of TSH Gene Expression.

Dr. Chin has continued to characterize both cis- and trans-acting elements involved in thyroid hormone negative regulation of TSH subunit gene expression. He has previously shown that the rat TSHβ subunit gene contains two transcriptional start sites with their associated TATA boxes located 43 bp apart. The transcript corresponding to the downstream start site appears to be most regulated by thyroid hormone. Dr. Chin has used deletion mutants and gene transfer into GH_3 cells to identify a 57 bp fragment, encompassing the downstream start site and the first exon, that can confer thyroid hormone–responsive negative regulation of the TSHβ gene. Furthermore, a protein-DNA–binding assay employing avidin-biotin complex formation has been used to locate two THR binding sites within this fragment at positions −13 to +4 and +11 to +27. These data indicate that two putative thyroid hormone–responsive elements (TREs) downstream of the rat TSHβ gene promoters may cooperate to permit thyroid hormone action. Similar work performed with the rat α-subunit gene has shown that its putative TRE is located at positions −74 to −53, in a region that is devoid of well-known proximal promoter elements, such as GC-rich or CAAT boxes. This region also binds THR as protein in nuclear extracts or as *in vitro* translation products. These data suggest that THRs may interact with DNA by direct binding, with interference of gene transcription on a steric basis.

II. Sex Steroid Hormone/Gonadal Peptide Regulation of LH and FSH Gene Expression.

Dr. Chin and his colleagues have shown that estrogen can regulate α, LHβ, and FSHβ mRNAs in pituitary glands of intact mature female and male rats. In addition, they have provided evidence that estrogen may have a direct effect on LH synthesis in the pituitary gland, in part by regulating the synthesis of LHβ and α mRNAs, with effects on subunit gene transcription. The 5′-flanking region of the rat LHβ gene was analyzed for a putative estrogen-response element. Such an element is present at −1,200 and confers positive regulation by estrogen on heterologous promoters. Another important point involves the differential regulation of FSHβ mRNAs by androgens. Androgens have little effect on FSHβ mRNA *in vivo* but markedly stimulate it *in vitro*. Dr. Chin has also shown that a number of gonadal peptides, including inhibin A and B, follistatin, and/or FSH-releasing peptide (activin) regulate FSHβ mRNA in a specific manner without effect on α or LHβ mRNA. In summary, the subunit-specific positive and negative regulation of gonadotropin gene expression by various gonadal factors and hormones may provide insight into the complex regulation of gonadotropins in the intact animal.

III. Thyroid Hormone Receptors.

The first reports of the isolation and characterization of cDNAs encoding putative THRs were provided by Drs. R. Evans and B. Vennstrom in late 1986. The former reported that a proto-oncogene, c-*erbA*, as expressed in human placenta, encodes a β form of THR, and the latter described a related cDNA from chick embryo that encodes an α form of THR. Soon thereafter it was shown that c-*erbA*α and c-*erbA*β are encoded by separate genes located on different chromosomes in humans. Thus there was early evidence of heterogeneity of mammalian THRs.

Studies on the molecular biology of THRs in the rat have been performed. In addition to identifying a cDNA encoding rat c-*erbA*α (410 aa; α-1) that was originally described by Dr. C. Thompson, a cDNA derived from a rat pituitary GH_3 cell line and encoding an isoform of rat c-*erbA*α (492 aa; α-2) was isolated. This form is nearly identical to α-1 in its first 370 amino acids, which encompass the entire DNA binding and most of the thyroid hormone–binding domains of the THR. The carboxyl-terminal 40 amino acids of α-1 (a region completely con-

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served between α-1 and β) is totally different from the corresponding region in α-2. This variation apparently results in the inability of α-2 to bind thyroid hormone or its analogues. However, this form retains the ability to bind TREs in a number of genes, including those encoding the rat growth hormone and TSH subunits. From these and other data it appears that the initial α-subunit gene transcript is differentially spliced to yield either the α-1 or α-2 mRNAs. Surprisingly, one of the spliced products is not a bona fide THR, because it lacks the ability to bind thyroid hormone. Similar rat α-2 forms were described by Dr. T. Mitsuhashi (brain) and Drs. S. Izumo and V. Mahdavi (heart). Dr. Chin showed that α-2 is the most abundant form in brain.

Dr. Chin has investigated the potential biological role of α-2 in the regulation of thyroid hormone–responsive genes. In particular, the ability of α-2 to bind putative TREs in such genes suggested a potential mechanism for interactions with thyroid hormone–binding forms. First, the presence of α-2 with α-1 in the clonal GH_3 cell line indicates the potential for the two forms to interact in the same cell. Second, in collaboration with Drs. Ronald Koenig, P. Reed Larsen (HHMI), and David Moore at Brigham and Women's Hospital and Massachusetts General Hospital, cotransfection experiments were performed in which α-2 was introduced with bona fide THRs into cells lacking functional receptors. The ability of each THR or related forms, separately or together, to transactivate genes was assessed. When a 5′-flanking region reporter gene construct was used, α-2 alone did not transactivate rat growth hormone gene expression. However, in combination with rat α-1 or human β, α-2 could inhibit the transactivation capability of these bona fide THRs. Thus it appears that two THR-related forms derived from a single transcript via alternate splicing possess competitive biologic actions. This suggests that α-2 might be a natural inhibitor of thyroid hormone activity.

In the study of the c-*erbA*α gene locus in the rat, the carboxyl-terminal protein-coding regions for α-1 and α-2 were shown to be encoded by adjacent exons. In fact, the penultimate 3′-exon of the rat c-*erbA*α gene encodes both a common region (i.e., a region that is identical in both α-1 and α-2) and a region specific for α-1. Hence, by alternate donor site choice, variable splicing can lead to either α-1 or α-2 in a tissue-specific manner. Further examination of the rat c-*erbA*α locus revealed yet another interesting finding. The transcripts in various tissues could be detected using a sense probe of a portion of the α-2–specific exon, suggesting transcription of the opposite strand. Such analyses revealed the presence of a gene (Rev-*erbA*α) that is transcribed from the opposite strand of the rat c-*erbA*α locus with the 3′-exons of the Rev-*erbA* and α-2 transcriptional units sharing 269 common bases that represent respective coding regions. Surprisingly, this opposite-strand transcript encodes another member (504 aa) of the c-*erbA* thyroid/steroid hormone receptor family, which also includes the retinoic acid and vitamin D receptors. Rev-*erbA*α, which does not bind thyroid hormone or TREs, is as related to THRs as it is to retinoic acid and vitamin D receptors. It is speculated that Rev-*erbA*α might be functional with an unknown ligand and undefined target genes, as well as the potential regulation of α-1/α-2 transcript production and mRNA translation via antisense nucleic acid mechanisms. The conservation of this genomic arrangement in humans and in rodents, as confirmed by this laboratory and by Dr. N. Miyajima, and the >95% homology between rat and human Rev-*erbA*α suggest the possible biological importance of this curious transcript and/or product.

Yet another putative THR form, β-2 (514 aa), was cloned from the rat pituitary GH_3 library. The β-2 is nearly identical to rat β-1 (461 aa) (homologue of human placental c-*erbA*β) from a region just amino-terminal to the DNA-binding domain through to the carboxyl terminus. The only difference is observed in the amino-terminal ends of the molecules involving the A/B region of members of the thyroid/steroid hormone receptor family. The β-2 is capable of trans-activating thyroid hormone–responsive genes but without apparent quantitative differences among the putative THRs. Remarkably, the β-2 mRNA is expressed only in the pituitary gland. This contrasts with the observation that α-1, α-2, and β-1 are expressed in nearly all other tissues, although in varying ratios. Thus marked tissue-specific expression of β-2 is notable.

This work has revealed the marked heterogeneity of THR and related forms. Indeed, there are at least three bona fide THRs, as judged by their ability to bind thyroid hormone and their analogues in the correct fashion and to transactivate putative thyroid hormone–responsive genes. A fourth variant form, α-2, related to α-1, may be a natural inhibitor of thyroid hormone action in various cells. The details of the role and regulation of the opposite-strand transcript encoding Rev-*erbA*α require more elucidation.

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

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MOLECULAR MECHANISMS OF SIGNAL TRANSDUCTION THROUGH CYCLIC NUCLEOTIDES

JACKIE D. CORBIN, PH.D., *Investigator*

Dr. Corbin's main interest is intracellular cyclic nucleotide receptors. Recent investigations have focused on three projects: 1) cAMP-dependent protein kinase, 2) cGMP-dependent protein kinase, and 3) negative feedback control of cyclic nucleotide levels.

I. cAMP-Dependent Protein Kinase (cAK).

cAMP sites of the cAK from the fungus *Mucor rouxii* have been characterized through the study of the effects of cAMP and cAMP analogues on the phosphotransferase activity and through binding kinetics. The tetrameric holoenzyme, which contains two regulatory (R) and two catalytic (C) subunits, exhibited positive cooperativity in activation by cAMP, suggesting multiple cAMP-binding sites. Several other results indicated that the *Mucor* kinase contained two different cooperative cAMP-binding sites on each R subunit, with properties similar to those of the mammalian cAMP-dependent protein kinase. Under optimum binding conditions, the [^{3}H]cAMP dissociation behavior indicated equal amounts of two components that had dissociation rate constants of 0.09 min^{-1} (site 1) and 0.90 min^{-1} (site 2) at 30°C. Two cAMP-binding sites could also be distinguished by C-8 cAMP analogues (site 1–selective) and C-6 cAMP analogues (site 2–selective); combinations of site 1– and site 2–selective analogues were synergistic in protein kinase activation. The two different cooperative binding sites were probably located on the same R subunit, since the proteolytically derived dimeric form of the enzyme, which contained one R and one C component, retained the salient properties of the untreated tetrameric enzyme. Unlike any of the mammalian cyclic nucleotide–dependent isozymes described thus far, the *Mucor* kinase was much more potently activated by C-6 cAMP analogues than by C-8 cAMP analogues. In the ternary complex formed by the native *Mucor* tetramer and cAMP, only the two sites 1 contained bound cAMP, a feature that has also not yet been demonstrated for the mammalian cAK.

II. cGMP-Dependent Protein Kinase (cGK).

A. *Structure of the cGMP-binding site(s).* Mammalian cAK and cGK show considerable similarity in amino acid sequence, although they specifically bind different cyclic nucleotides. Results of cGMP analogue-binding experiments, combined with modeling of the cGMP-binding sites by analogy to the structure of the homologous catabolite gene activator protein, suggest that a threonine residue forms a hydrogen bond with the 2-NH_2 of cGMP. This threonine is invariant in all cGMP-binding domains, but the corresponding residue in 23 out of 24 cAMP-binding sites of protein kinases is alanine, which cannot form the same hydrogen bond. This alanine/threonine difference has the potential for discriminating between cAMP and cGMP and may be important in the evolutionary divergence of cyclic nucleotide–binding sites.

B. *Monomeric cGK.* A form of cGK that was different from previously described cGK was purified from bovine aorta smooth muscle. The partial amino-terminal sequencing of this enzyme indicated that it was derived by endogenous proteolysis of the type Iβ isozyme of cGK. On SDS-PAGE, this form migrated as a smaller protein (M_r = 70,000) than the parent cGK (M_r = 80,000), and since the calculated nondenatured M_r was ~89,000 compared with M_r = 170,000 for the dimeric native enzyme, it represented a monomeric form of cGK. The monomer bound ~2 mol of [^{3}H]cGMP per mole of monomer, although it had only one rapid component in [^{3}H]cGMP dissociation assays, as compared with one rapid and one slow component for the native cGK. The specific catalytic activity of the kinase was similar to that of the native enzyme, suggesting that the catalytic domain was essentially intact. The monomeric cGK incorporated significant ^{32}P when incubated with Mg^{2+} and [γ-^{32}P]ATP in the presence of cGMP, although the phosphorylation proceeded at a slower rate than that obtained with native cGK. In contrast to previous reports of monomeric forms of cGK, this monomer was highly cGMP dependent, although it had a slightly higher K_a (0.8 μM) for cGMP than that of the native enzyme (0.4 μM) and a lower Hill coefficient (1.0 versus 1.6 for the native enzyme). The cGMP dependence of the monomer did not decrease with dilution, implying that the cGMP dependence was not due to monomer-monomer interactions in the assay. The results indicated that the catalytic domain, cGMP-binding domain(s), and inhibitory domain of cGK interact primarily within the same subunit rather than between subunits of the dimer, as previously hypothesized for dimeric cGK.

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III. Negative Feedback Control of Cyclic Nucleotide Levels.

The purified C subunit of cAK produced a two-fold activation of the low K_m phosphodiesterase in crude microsomes (P-2 pellet) of rat adipocytes. This activation was C subunit–concentration dependent, ATP dependent, blocked by a specific peptide inhibitor, and lost if the C subunit was first heat-denatured. The concentration of ATP necessary for half-maximal activation of the low K_m phosphodiesterase was 4.5×1.1 μM, which was nearly the same as the known K_m of C subunit for ATP (3.1 μM) using other substrates. The concentration of C subunit producing half-maximal activation of phosphodiesterase was 0.22×0.04 μM, slightly less than the measured concentration of total C subunit in adipocytes (0.45 μM). The activation of the low K_m phosphodiesterase by C subunit was specific, since on an equimolar basis, myosin light chain kinase, cGK, or Ca^{2+}/calmodulin-dependent protein kinase II did not activate the enzyme. The percent stimulation of phosphodiesterase by C subunit was about the same as that produced by incubation of adipocytes with a cAMP analogue, and the enzyme first activated *in vivo* with the analogue was not activated to the same extent (on a percentage basis) by *in vitro* treatment with C subunit. Treatment of the crude microsomes with trypsin resulted in transfer of phosphodiesterase catalytic activity from the particulate to the supernatant fraction, but the enzyme in the supernatant was minimally activated by C subunit, suggesting either loss or dislocation of the regulatory component. The C subunit–mediated activation of phosphodiesterase was preserved after transfer of phosphodiesterase activity to the supernatant fraction by non-ionic detergents or partial purification of the transferred enzyme. These findings are consistent with the suggestion that protein kinase regulates the concentration of cAMP through phosphodiesterase activation and provide direct evidence that the mechanism of activation involves phosphorylation.

Dr. Corbin is Professor of Molecular Physiology and Biophysics at the Vanderbilt University School of Medicine.

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MOLECULAR MECHANISMS AND VASCULAR DISEASE

SHAUN R. COUGHLIN, M.D., PH.D., *Assistant Investigator*

This year has represented a major change in direction for Dr. Coughlin's laboratory, with the addition of two new projects.

I. Molecular Mechanisms of Thrombin Action.

Dr. Coughlin's laboratory is pursuing a program to identify 1) the structural determinants in thrombin required for cell activation and 2) the thrombin "receptor" on platelets and other cells.

A. *Thrombin biology.* Thrombin is a multifunctional serine protease. It is produced from the circulating zymogen prothrombin by cleavage on cell and platelet surfaces when active prothrombinase complexes are present, as occurs in vascular injury. The thrombin concentrations reached during blood clotting are more than sufficient to elicit various cellular responses to thrombin.

In addition to its well-known role in cleaving fibrinogen to fibrin during blood coagulation, thrombin has a number of important cell-activating functions. Thrombin is the most potent physiological stimulator of platelet aggregation, and recent studies suggest that thrombin's actions are critical for platelet plugging and arterial thrombus formation. A number of the actions of thrombin on other circulating cells and on the component cells of the blood vessel wall are potentially important. Thrombin is a potent mitogen for lymphocytes and is chemotactic for monocytes. Thrombin elicits a number of responses from vascular endothelial cells, including triggering expression of adhesive molecules for neutrophils and the potent smooth muscle cell mitogen platelet-derived growth factor (PDGF). Thrombin is also a potent mitogen for vascular smooth muscle cells and fibroblasts. Tissue factor expression by macrophages in atherosclerotic plaques has recently been reported; it is not known whether local intramural production of thrombin occurs outside of the context of blood clotting or whether it plays a role in vascular proliferative or inflammatory responses. Thus the cell-activating functions of thrombin appear to be critical for normal hemostasis and may play an important role in proliferative and inflammatory responses in the vessel wall.

The mechanisms by which thrombin activates platelets and other cells are unknown, and the cell surface receptor that mediates thrombin action has not been identified. An understanding of these mechanisms is likely to reveal novel signal transduction mechanisms. In addition, because thrombin binding to cells and platelets does not reflect binding to the functional thrombin receptor, identification of the receptor and its mode of activation will be important for the development of therapeutics directed at the thrombin receptor.

B. *Thrombin mechanisms.* Dr. Coughlin's laboratory is producing recombinant thrombins that address thrombin's mechanism of action. One set of mutant thrombins addresses the requirement of thrombin protease activity for activation of cells and platelets. There is considerable debate as to whether thrombin activates cells by cleaving its receptor or by a more classical binding mechanism. The goal is not only to answer this mechanistic question but also to produce novel reagents for the study and purification of the thrombin receptor. Studies with recombinant trypsins have shown that it is possible to ablate protease activity by amino acid substitutions at catalytic triad residues; these mutant trypsins were catalytically inactive but were able to bind substrate normally. In collaboration with Dr. Charles Craik, Dr. Coughlin was able to show that the mutant trypsins would block the action of wild-type enzyme by competing for substrate. Dr. Coughlin's laboratory then developed an expression system for prothrombin and used ecarin, a snake venom protease, to convert the recombinant prothrombin to active thrombin. Methods to purify and quantitate prothrombin protein were also developed. The active-site mutations analogous to those described for trypsin have been introduced into the prothrombin cDNA. Once the mutant thrombins are expressed, their ability to activate cells and platelets will be assessed. If proteolytically inactive thrombin is capable of acting as a thrombin agonist, it will prove the occupancy theory of thrombin activation. If the proteolytically inactive mutant thrombin does not act as an agonist, it will be necessary to demonstrate that it still binds to the receptor to conclude that proteolytic activity is required for activation. To demonstrate binding, Dr. Coughlin will assess the ability of the mutant to act as a thrombin antagonist. Preliminary studies with trypsin mutants suggest that the analogous mutant thrombins will still bind their receptor, and a definitive answer to the activation by binding ver-

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sus proteolysis question will be possible. Proteolytically inactive thrombins that bind the thrombin receptor would also be invaluable as affinity reagents for receptor purification.

C. *Thrombin receptor.* Previous attempts to identify the thrombin receptor have not succeeded. Binding and crosslinking studies have been confounded by a number of high-affinity thrombin-binding proteins involved in terminating thrombin's action rather than mediating it. In addition, the nature of thrombin's interaction with its receptor (occupancy versus proteolysis) is not known. Several strategies have been adopted to identify and obtain the cDNA for the thrombin receptor. The first strategy, expression cloning in *Xenopus* oocytes, requires no assumptions regarding the nature of thrombin's interaction with its receptor. The only criterion is that the molecule cloned by this technique must specifically confer thrombin responsiveness on the oocytes; i.e., it is a thrombin receptor. Dr. Coughlin's laboratory first screened a number of mammalian cell lines for thrombin responsiveness using calcium mobilization, an early biochemical response to thrombin stimulation, as an endpoint. HEL cells (a human megakaryocyte-like cell line) demonstrated a particularly robust response. Dr. Coughlin's laboratory then showed that microinjection of *Xenopus* oocytes with mRNA prepared from HEL cells conferred thrombin responsiveness on the oocytes (in this case, thrombin-induced increases in calcium mobilization were assessed by thrombin-induced increases in ^{45}Ca efflux from prelabeled oocytes). Size fractionation of the HEL mRNA suggested that the thrombin receptor is encoded by a single mRNA species of ~4 kb. These experiments establish that oocytes will translate an mRNA species encoding the thrombin receptor and properly process the product. Moreover, the signal transduction machinery necessary to couple this receptor to a response is present and functional in the oocytes.

The oocyte expression system thus constitutes an assay for thrombin receptor mRNA. This can be exploited to obtain a cDNA clone encoding the receptor. This general strategy has been successful for the serotonin receptor and substance K receptors. A cDNA library is made in a vector that allows *in vitro* transcription of the cDNA insert. The library is divided into pools of a specific complexity (e.g., 20,000 clones/pool). DNA from each pool is prepared and transcribed *in vitro*. The resulting synthetic mRNA is assayed for receptor activity in the oocyte system. Once a positive pool is found, it is plated at a lower complexity (e.g., 1 pool of 20,000 would be divided into multiple pools of 2,000). DNA from each of these pools is then transcribed, and the resulting mRNA is again assayed in the oocyte system. By repeated iterations, a single clone encoding the receptor is obtained. This tedious approach has the advantage that once a positive pool is found, the path to the receptor clone is straightforward. By virtue of the selection method, the molecule that is obtained will be the functional receptor.

Dr. Coughlin's laboratory has characterized the screening system with a model phage containing cDNA encoding the serotonin receptor (5HT1c); these studies established that the screening method was sensitive enough to detect a single serotonin receptor clone among 20,000 other phage. Two HEL cDNA libraries and one endothelial cDNA library are currently being screened.

An alternative approach to expression cloning, using novel recombinant thrombins as affinity reagents to purify the receptor, is also planned.

Once a cDNA clone is obtained, the following questions will be addressed: 1) Is the receptor cleaved by thrombin, and what is the relationship of cleavage to activation? 2) Do other thrombin receptor-like molecules exist? Does the thrombin receptor define a family of protease receptors? Where are they expressed? 3) What sequences within thrombin and its receptor mediate thrombin-receptor interaction? Can a soluble receptor extracellular domain or portions thereof be used to block the platelet activation by thrombin? 4) What signal transduction molecules associate with the thrombin receptor and mediate its actions?

II. Cells and Molecules Mediating Graft Atherosclerosis.

Dr. Coughlin's laboratory has established a consortium to study the pathogenesis of accelerated transplant atherosclerosis as one model of pathological vascular smooth muscle cell proliferation. The laboratories of Drs. Bruce Hall, David Gordon, and Josiah Wilcox are participating.

Accelerated transplant atherosclerosis in the coronary arteries of transplanted hearts is the major limitation to long-term survival of transplant recipients. The disease is distinct from nontransplant atherosclerosis in its tempo (at least 50% have significant disease within 5 years) and its pathology (a diffuse concentric proliferative lesion that extends

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longitudinally along the epicardial coronaries and into the penetrating branches). The premise of the studies is that accelerated transplant atherosclerosis represents an interaction of the recipient's immune cells with the growth factor systems governing proliferation of vascular smooth muscle cells. A rat model of accelerated transplant atherosclerosis has been established, and probes for a number of rat growth factors and growth factor receptors have been cloned. Immunohistochemistry and *in situ* hybridization techniques are being used to identify the cells (T cells, B cells, monocytes) and molecules (growth factors, cytokines, and their receptors) that are present during the genesis of accelerated atherosclerosis in the rat model. Spatial and temporal correlations with cell proliferation (identified by immunohistochemical staining for cyclin) in the artery wall will be made. Cell culture models of the interactions of lymphocytes with endothelial and vascular smooth muscle cells are being developed.

Dr. Coughlin is also Assistant Professor of Medicine at the University of California at San Francisco.

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GENETIC REGULATORY EVENTS IN T LYMPHOCYTE ACTIVATION

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

Work in the Crabtree laboratory centers around molecular mechanisms of cellular differentiation. The fundamental events involved in this process are studied using two systems: T lymphocyte activation and the developmental control of the hepatic phenotype. Work on T lymphocyte activation is funded by the Howard Hughes Medical Institute.

T lymphocyte activation refers to the sequential morphologic and functional changes that culminate in cell proliferation and immune function after a quiescent T cell is exposed to its cognate antigen in the proper histocompatibility context. Because early studies indicated that only 30–60 min of exposure to lectins is required for commitment to T cell activation, attention has been focused on these early events after antigen interacts with the antigen receptor. The hypothesis that guides this work is that essential genetic programming events are set in motion during this time. Understanding the molecular nature of these programming events is the object of these studies.

I. Identification of Nuclear Targets for the T Lymphocyte Antigen Receptor.

Because signals from the antigen receptor govern the decision to initiate the activation pathway or remain in a quiescent state, Dr. Crabtree and his colleagues began a number of years ago to define nuclear targets for the antigen receptor. They chose the interleukin-2 (IL-2) gene to search for such nuclear targets, since 1) the IL-2 gene is rigorously controlled by the antigen receptor, 2) expression of IL-2 is essential for proliferation and immunologic activation, and 3) transcription of the gene is initiated at the time that T cells become committed to proliferation and immunologic function. These studies led to the identification of a transcriptional enhancer lying just 5′ of the IL-2 gene that mediated T cell specificity, antigen-receptor inducibility, and inhibition by cyclosporin and glucocorticoids.

The precise sequences required for the action of this enhancer were defined by examining the function of a series of linker-scanner and internal-deletion mutants within the enhancer transfected into T cell lines. These studies led to the recognition of three short sequences of 10–20 bp that were essential for full function of the enhancer. The role of these sequences in the function of the IL-2 enhancer was supported by studies in which these sequences were synthesized and several copies attached to an unrelated promoter. Two of these synthetic sequences, in two, three, or four copies, activate transcription of an unrelated promoter in response to signals from the T cell antigen receptor; hence they were called antigen receptor response elements (ARRE-1 and ARRE-2).

A. *A novel function for the Oct-1 transcription factor in T cell activation.* The most 3′-proximal ARRE binds a protein found in all cell types examined to date. The protein binding to this site was initially called NFIL2-A. More recently, this protein has been purified and found to have the same molecular weight, sequence specificity, and antigenic determinants as the previously identified Oct-1 transcription factor. In addition, proteolytic digestion fragments generated with V-8 protease and Arg C protease are identical, strongly indicating that NFIL2-A is identical to Oct-1. Because tandem repeats of this site activate transcription within 15–20 min after triggering the antigen receptor and activation by this site does not require protein synthesis, the group now believes that the Oct-1 transcription factor is post-translationally modified or binds a second protein that is post-translationally modified. These results, which indicate that Oct-1 is transmitting signals directly from the antigen receptor, will facilitate the approach to understanding how signals from the antigen receptor are transmitted to the nucleus. In the coming year the major objective of the laboratory will be to characterize the way in which the Oct-1 protein becomes active in response to signals initiated at the antigen receptor.

B. *NFAT protein appears to account for the tissue specificity and protein synthetic requirement for early gene activation.* The most 5′ functional site in the IL-2 enhancer binds a protein that has been found only in nuclear extracts of activated T cells. This protein, nuclear factor of activated T cells (NFAT), appears ~15–20 min after T cells are activated with lectin and precedes the first detection of IL-2 mRNA by 10–20 min. The appearance of the binding activity requires the activation of an earlier gene. In footprinting and competition studies, NFAT or a very similar protein binds to sequences important for transcriptional activation of the long-terminal repeat (LTR) of human immunodeficiency virus (HIV). Thus NFAT-1 may be a general regula-

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tor of early T cell activation genes. To test this hypothesis, the laboratory has prepared transgenic mice with three copies of the 28 bp NFAT-binding site directing transcription of several indicator genes. In four of four transgenic lines in which the NFAT site was present, properly initiated transcription of the indicator gene was restricted to activated lymphocytes, whereas in transgenic lines without the NFAT site, transcription was either absent or present in inappropriate tissues. These results indicate that the NFAT protein accounts at least partly for the remarkable tissue specificity of IL-2 expression. In the coming year, Dr. Crabtree and his colleagues will attempt to purify and characterize the NFAT protein.

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

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MEMBRANE PROTEIN STRUCTURE/FUNCTION

LELAND ELLIS, PH.D., *Assistant Investigator*

The response of cells to the polypeptide hormone insulin begins with the binding of insulin to a specific cell surface receptor. The insulin receptor (IR) is synthesized as a large (1,355 amino acid) single polypeptide chain precursor, which undergoes post-translational processing to yield an integral transmembrane glycoprotein comprising two α-subunits (~135 kDa) and two β-subunits (~95 kDa). Dr. Ellis and his colleagues wish to relate the structural features of the IR protein to the details of IR function. The size and relatively simple transmembrane topology of the IR make it possible to engineer and study its two functional domains individually as soluble molecules: the extracellular domain is secreted as a dimer that binds insulin with high affinity, and the cytoplasmic domain is an active monomeric protein tyrosine kinase (PTK). Ideas and hypotheses concerning receptor structure/function have derived from this reductionist approach. Transfected mammalian cell lines and transgenic mice are being used to test these ideas in the context of the intact transmembrane receptor (in collaboration with Dr. Robert Hammer, HHMI, University of Texas Southwestern Medical Center at Dallas).

The extracellular ligand-binding domain of the IR is a complex molecule: each half of the disulfide-linked $(\alpha\beta)_2$ dimer comprises 929 residues derived from both α- (735 aa) and β-subunits (194 aa), with 16 potential N-linked glycosylation sites and 41 cysteines. Furthermore, little is presently known about how this domain folds during biosynthesis or how this domain interacts with insulin. The study of an extensive series of deletion mutants has revealed the location within the primary amino acid sequence of independently folded soluble subdomains, as well as their stability, efficiency of secretion, and interaction with both insulin and a panel of monoclonal antibodies specific for the extracellular domain of the receptor. These landmarks now guide the future biochemical and molecular dissections of this domain.

The baculovirus insect cell (Sf9) system has been successfully employed to express an active soluble derivative of the cytoplasmic IR PTK domain. Results demonstrate the utility of this approach for the study of functional domains of large membrane proteins. The availability of milligram quantities of the protein now renders feasible the use of biophysical methods such as circular dichroism (CD) and nuclear magnetic resonance (NMR) spectroscopy to study the interaction of small molecules (metal ions, ATP, peptide substrates) with the enzyme in solution. Thus new avenues are available with which to explore the function of the enzyme. The NMR studies (in collaboration with Dr. Barry Levine, Oxford University) have provided the first look at catalysis by a PTK in real time, as it is now possible to follow in solution the binding of peptide substrates to the enzyme and the phosphorylation of individual tyrosine residues of peptide substrates (especially the order of phosphorylation of multiple tyrosines in peptide substrates). The role of individual amino acid residues of the peptide on its binding to the enzyme and its kinetics of phosphorylation can also be studied. These functional studies complement efforts to obtain the three-dimensional structure of the enzyme by x-ray crystallography (in collaboration with Dr. Wayne A. Hendrickson, HHMI, Columbia University College of Physicians and Surgeons).

Dr. Ellis's interest in membrane proteins and their structural organization stems from a general interest in cell membranes and cell-cell interactions, especially in the developing nervous system. After the cessation of mitosis, neurons enter a stage of development during which cell processes (axons and dendrites) are elaborated and connections (synapses) with appropriate target cells are established. Such elongating processes end in a terminal enlargement, the nerve growth cone. The sprouting neuron responds to a number of extracellular signals, including growth factors and hormones, components of the extracellular matrix, and its target cell. The biochemical and molecular components of the neuronal plasmalemma (especially the nerve growth cone) that mediate(s) such critical developmental events must be described before this stage of neuronal differentiation can be understood.

The growth cone particle (GCP) fraction prepared from fetal (17 day gestation) rat brain consists of pinched-off cellular fragments that exhibit all of the ultrastructural features of nerve growth cones. GCPs are utilized to identify and characterize membrane-associated proteins expressed during neuronal sprouting. The initial biochemical analysis of membranes prepared from GCPs revealed three major polypeptides: pp46, p38, and p34. Subsequently pp46 was shown by others to be the growth-associated protein, GAP-43, a protein of un-

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known function that is expressed during neuronal sprouting (both *de novo* and during regeneration). Both p38 and p34 are intimately associated with GCP membranes (as they partition into the detergent phase after TX-114 extraction), and their relative abundance provided sufficient amounts for gas-phase protein microsequencing (in collaboration with Dr. Clive A. Slaughter, HHMI, University of Texas Southwestern Medical Center at Dallas). Multiple tryptic peptides from each protein were sequenced and revealed that p38 and p34 correspond to the α- and β-subunits of G proteins, respectively. This identification was confirmed for each by the use of subunit-specific antipeptide antibodies (courtesy of Dr. Alfred G. Gilman, University of Texas Southwestern Medical Center at Dallas). Thus the major polypeptides associated with GCP membranes (pp46/GAP-43, p38/G_α, or p34/G_β) are peripheral proteins associated with the cytoplasmic surface of the neuronal plasmalemma, not integral membrane proteins. Larger scale biochemical preparations of GCP membranes, together with immunological strategies, will be required to identify other more minor components.

To complement these biochemical studies of nerve growth cone membranes, Dr. Ellis and his colleagues have initiated a second approach to identify genes expressed at the onset of neural sprouting. In collaboration with Dr. Hammer, a reporter gene (bacterial β-galactosidase) linked to the weak herpes simplex virus thymidine kinase promotor (i.e., a promotor devoid of tissue-specific elements and therefore subject to positional effects) has been introduced into the germline of transgenic mice to mark areas of active transcription during different stages of mouse development. Histological examination of whole fetuses and sections of transgenic offspring at different stages of embryonic and fetal development identify those lines that express the reporter gene in areas of interest in the central nervous system at the time of neuronal sprouting. The DNA of the transgene (of viral/bacterial origin) will be used for cloning of the flanking regions of host mouse DNA for the characterization of the enhancer elements and structural genes interrupted by the integration event. This strategy complements the study of the GCP and obviates the limitations of protein quantity or antigenicity inherent in a biochemical/immunological approach.

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

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BIOCHEMISTRY AND PHYSIOLOGY OF THE PROTEIN C ANTICOAGULANT PATHWAY

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

I. Thrombomodulin-Thrombin Interaction.

Thrombomodulin is an integral membrane protein that binds thrombin and accelerates the activation of protein C more than 1,000-fold. The binding interaction also inhibits the capacity of thrombin to clot fibrinogen. The overall structure of thrombomodulin is reminiscent of the low-density lipoprotein (LDL) receptor, with an amino-terminal domain, a series of six growth factor repeats, an O-linked sugar region, a transmembrane domain, and a cytosolic tail. Limited digestion of thrombomodulin with elastase generated a soluble, active form of thrombomodulin that was composed essentially only of the growth factor repeats. CNBr digestion revealed that residues 310–486 were all that were required for acceleration of thrombin-catalyzed protein C activation and that a small domain corresponding to the carboxyl-terminal 80 residues of this fragment contained the major thrombin-binding site. Although this fragment could not accelerate protein C activation, it retained the capacity to inhibit fibrinogen clotting.

With the identification of the thrombin-binding site, new questions were posed about the topographical distribution of thrombin, thrombomodulin, and the membrane surface. These questions were addressed by fluorescence energy transfer between a dansyl moiety in the active center of thrombin and octadecylrhodamine in the membrane. The active center of thrombin was located ~65 Å above the membrane. Thus the O-linked sugar domain must rise essentially vertically from the membrane surface. The distance to the membrane surface of thrombin in the thrombin-thrombomodulin complex is almost identical to that found when the distance is measured in prothrombin. Therefore, although thrombomodulin and the prothrombin activation fragments have almost no sequence similarity, the two form similar "platforms" on which the enzyme rests.

The interaction with thrombomodulin also alters the fluorescence properties of the dansyl dye in the active center of thrombin, supporting the concept that thrombomodulin alters the catalytic properties of thrombin by altering the conformation of thrombin.

II. Factor V Structure and Function.

Factor V is a large (300 kDa) plasma protein that is proteolytically modified by thrombin to an active form Va composed of two nonidentical subunits held together in a calcium-dependent interaction. Previous studies indicated that only a single Ca^{2+} is involved in the interaction and that the kinetics of association are extremely slow and very temperature dependent. These properties suggested that major conformational changes might be involved in the reassociation process. Fluorescence and circular dichroism studies were used to investigate the extent of structural changes in factor Va during reassociation. The results indicated that the structural changes, although reproducible, were small and correlated with the formation of biological activity. Thus the original hypothesis that the slow and very temperature dependent reassociation was due to major conformational changes seems unlikely.

Although the sequence of factor V is known, relatively little is known about the location of specific binding sites on the protein or the mechanisms by which they function. Studies in collaboration with Drs. Arthur Johnson and Thomas Laue have revealed that binding factor Va to both factor Xa and prothrombin alters the distance to the membrane surface. Thus one potential mechanism involved in factor V function may be to align the proteolytic center of factor Xa with the activation site on prothrombin. Previous studies from Dr. Esmon's laboratory indicated that the major prothrombin-binding site on factor Va was on the heavy chain of the heterodimer. Direct equilibrium binding interactions were undertaken using the analytical ultracentrifuge. These studies confirmed and extended the previous qualitative observations. The heavy chain forms a Ca^{2+}-independent, 1:1 complex with prothrombin that is governed by a dissociation constant of 10 μM. The cleavage of the heavy chain with activated protein C abolishes all detectable association of the heavy chain with prothrombin. These findings support the concept that the regulatory proteins in coagulation function in part by binding to the substrates.

III. Physiological Studies.

The protein C pathway appears to be modulated by inflammatory mediators. During septic shock, the levels of C4b-binding protein (C4bBP) rise, and this serves to complex protein S and inhibit the anticoagulant pathway. This raised the question of

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whether this elevation in C4bBP directly contributed to the organ damage found in the shock process. To resolve this question, the C4bBP levels were elevated to equal those found in patients with disseminated intravascular coagulation (DIC) and challenged with a sublethal level of bacteria that normally only causes the acute-phase response and no DIC. Elevation of the C4bBP levels resulted in DIC and organ failure. This response could be reversed in part by elevating protein S. These studies indicate that reversing the C4bBP–protein S complex formation would aid in the treatment of gram-negative sepsis.

Previous studies from Dr. Esmon's laboratory indicated that activated protein C can protect animals from gram-negative shock. Since activated protein C is an anticoagulant, the question arises as to whether this protection from septic shock is due to the capacity to block clotting. To resolve this question, Dr. Esmon designed a new anticoagulant based on knowledge that factor Xa binding to factor Va was independent of the active center of factor Xa. Therefore factor Xa was inhibited with a specific chloromethyl ketone. This inactive factor Xa worked as an anticoagulant both *in vitro* and *in vivo*, presumably by competing with factor Xa for binding with factor Va on cellular surfaces. Although the active site–blocked factor Xa was more effective than activated protein C in blocking DIC, this inhibitor was completely without effect in preventing organ damage. Thus the action of activated protein C appears to be other than the capacity to inhibit fibrin formation. Ultimately these studies should provide a much better rationale for the treatment of septic shock. This work was conducted in collaboration with Dr. Fletcher Taylor.

It is well recognized that thrombus formation in the coronary artery is responsible for most heart attacks. The role of clotting or protein C in ischemic injury is not well understood. In collaboration with Dr. Thomas Snow, studies were initiated to investigate the influence of activated protein C or the inhibition of activated protein C on recovery after coronary artery occlusion. The results show that blocking protein C activation increases the frequency of fibrillation after reperfusion and that activated protein C infusion increases the rate of recovery of cardiac function.

Dr. Esmon is also Member of the Cardiovascular Biology Research Program at the Oklahoma Medical Research Foundation and OMRF Associate Professor of Biochemistry and of Pathology at the University of Oklahoma Health Sciences Center.

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CELL SIGNALING THROUGH PHOSPHOLIPID BREAKDOWN

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

I. Agonist and Guanine Nucleotide Regulation of Polyphosphoinositide Phospholipase C.

It is commonly accepted that many hormones and neurotransmitters exert their effects on their target cells by stimulating the breakdown of a plasma membrane phospholipid, phosphatidylinositol 4,5-bisphosphate (PIP_2), through activation of a specific phospholipase C to yield two intracellular signaling molecules, namely inositol 1,4,5-trisphosphate (IP_3), which releases Ca^{2+} ions from an internal store, 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 GTP-binding regulatory protein (G protein) that links these. The V_1-vasopressin receptor has been solubilized from liver plasma membranes and purified to apparent homogeneity; a key step in this process is affinity chromatography on a V_1-vasopressin antagonist linked to agarose. The receptor, which can be specifically crosslinked to [^{125}I]vasopressin using disulfosuccinimidyl tartrate, is a 58 kDa protein that shows a selectivity for V_1 antagonists compared with V_2 antagonists. Peptides derived from the receptor are being sequenced to develop probes to begin cloning.

Two PIP_2 phospholipases have been identified in liver plasma membranes; one of them is apparently regulated by $G_{p\alpha}$ (see below) and has been purified to near homogeneity. This 148 kDa polypeptide cross-reacts with monoclonal antibodies to the type II or γ form of the enzyme from brain.

Several G proteins have been purified from rat liver plasma membranes as α β γ heterotrimers. Some of them have been identified as G_i and G_s species by toxin labeling and reconstitution with SV9 cyc$^-$ membranes, whereas others represent novel G proteins. Purification of some of these unidentified G proteins to homogeneity is being undertaken. The purified G proteins and their resolved α-subunits are being analyzed by labeling with [^{32}P]azidoanilido GTP and by Western analysis, using polyclonal antisera to different peptide sequences in the α-subunits of known G proteins (G_{i1}, G_{i2}, G_{i3}, G_o, and T_d) and to a sequence common to most G protein α-subunits. They will be tested for the activity of G_p (the G protein regulating PIP_2 phospholipase C) by 1) reconstitution with preparations of the vasopressin receptor and measurement of GTPase activity and GTP effects on vasopressin binding; 2) reconstitution with PIP_2 phospholipase C and assay of GTPγS stimulation of activity; and 3) reconstitution with liver plasma membranes photolyzed with azidoanalido GTP, to inactivate endogenous G proteins, and assay for GTPγS stimulation of endogenous PIP_2 phospholipase C.

In another study, G protein α-subunits are being solubilized from liver plasma membranes previously treated with a GTP analogue (GTPγS), and are being purified to identify $G_{p\alpha}$ (the α-subunit of G_p). With this protocol a protein (presumably $G_{p\alpha}$) has been shown to retain its ability to activate PIP_2 phospholipase C through several chromatographic steps. This protein is being purified to homogeneity to provide partial sequence to begin cloning.

II. Regulation of Phosphatidylcholine Breakdown in Isolated Hepatocytes.

High-performance liquid chromatography (HPLC) analyses of the molecular species of DAG generated in hepatocytes in response to Ca^{2+}-mobilizing agonists have indicated that only part of the DAG accumulating in response to hormones is derived from the breakdown of phosphoinositides and that there is another major source. Fatty acid analyses of the DAG species have shown that the most likely source is phosphatidylcholine (PC). When this phospholipid is selectively labeled with [^{3}H]alkyl-lyso-glycerophosphocholine or [^{14}C]lyso-PC, its breakdown to DAG and phosphatidic acid (PA) is stimulated by Ca^{2+}-mobilizing agonists. The response is mimicked by the Ca^{2+} ionophore A23187 and is markedly reduced in Ca^{2+}-depleted cells, implying that it is secondary in part to the elevation in cytosolic Ca^{2+} induced by the agonists. The breakdown of labeled PC is also induced by tumor-promoting phorbol esters, suggesting the involvement of protein kinase C. The molecular DAG species generated by A23187 are similar to those produced by the Ca^{2+}-mobilizing agonists but differ from those induced by phorbol ester. This suggests that the mechanisms involving Ca^{2+} and protein kinase C are not identical. Current efforts are directed toward defining the specific phospholipases and other components involved in both mechanisms of regulation of PC hydrolysis. As a first approach, the characteristics and subcellular distributions of PC

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phospholipase C, PC phospholipase D, PA phosphohydrolase, and DAG kinase are being defined in rat liver.

A sensitive method for the measurement of PA in tissues has been developed and used to determine the changes in PA in regenerating liver after partial hepatectomy. This lipid increases significantly 0.5 h after surgery and reaches a maximum value at 1.5 h. In contrast, DAG increases maximally at 0.5 h and then declines. These early changes in DAG and PA may play signaling roles in the acceleration of growth after hepatectomy.

A protein kinase that is stimulated by PA has been identified in rat liver cytosol. It selectively catalyzes the phosphorylation of 33 and 35 kDa proteins and also some of higher and lower weights. The stimulation by PA depends on Ca^{2+} in the submicromolar (i.e., cytosolic) range and is not mimicked by other lipids, including DAG in combination with phosphatidylserine; i.e., it does not involve protein kinase C. The PA-stimulated kinase is active toward histone 1, histone 1A, and myelin basic protein. When tested against the specific peptide substrates of known protein kinases, it is most active toward a peptide of the S6 ribosomal protein but causes little phosphorylation of the peptide substrates of cAMP-dependent protein kinase, cGMP-dependent protein kinase, Ca^{2+}-calmodulin–dependent protein kinase II, protein kinase C, and several protein tyrosine kinases. The PA-stimulated protein kinase is being purified to homogeneity. It may be an important mediator of the cellular effects of PA.

Dr. Exton is also Professor of Molecular Physiology and Biophysics and of Pharmacology at Vanderbilt University School of Medicine.

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ELUCIDATING THE MOLECULAR MECHANISMS UNDERLYING EPIDERMAL GROWTH, DIFFERENTIATION, AND DEVELOPMENT IN HUMAN SKIN

ELAINE FUCHS, PH.D., *Associate Investigator*

The long-range objective of this laboratory is to understand the regulation of the expression of human genes during differentiation and development in epithelial tissues, particularly those of the skin. An understanding of the biochemical mechanisms underlying differentiation is a prerequisite to elucidating how these processes go awry in epithelial diseases. Epithelial tissues have a common protective function, which is manifested by the production of an extensive cytoskeletal architecture. The unique 8 nm keratin filaments of this framework account for 1–5% of the protein in simple epithelial tissues, 30% of the protein in mitotically active epidermal cells, and up to 85% of the protein in fully differentiated epidermal squames and hair cells.

There are more than 20 different keratins. These keratins can be subdivided into two types (I and II), both of which are essential for filament assembly. Keratins are frequently expressed as specific type I and type II pairs, and the pattern of pairwise expression varies with epithelial cell type and with relative stages of differentiation and development. Expression of keratins is also sensitive to the cellular environment, suggesting that keratins are tailored to suit the varied structural and functional needs of each epithelial cell.

I. Regulation of Epidermal Differentiation by Vitamin A.

Previously this laboratory has optimized conditions for cultivation of human epidermal cells; most of their differentiative functions, including stratification and expression of the differentiation-specific keratins K1 and K10, are maintained. In the past year these methods were extended to produce two additional model systems: one for studying transformation of keratinocytes by human papillomaviruses and one for studying squamous cell carcinomas (SCCs) *in vitro*. For both systems the morphological and biochemical features of abnormal differentiation are very similar to those that occur in the disease state *in vivo*.

By culturing normal epidermal and SCC cells on floating collagen-fibroblast lattices, the laboratory has investigated the effects of vitamin A and its analogues on normal and abnormal differentiation and on growth. Normal epidermal cultures produce a single layer of mitotically active basal cells that express the type I keratin K14 and the type II keratin K5. As these cells undergo a commitment toward terminal differentiation, they downregulate the expression of these basal keratins and induce the expression of keratins K10 and K1. SCC-13, a cell line from a human squamous cell carcinoma of the skin, produces 2–3 layers of basal cells, with a much greater proportion of cells in S phase (DNA synthesis) than normal. As these cells undergo a commitment toward terminal differentiation, they downregulate the expression of K5 and K14 and induce the expression of K6 and K16, a pair of keratins not normally expressed in the epidermis *in vivo* but induced in a variety of epidermal diseases associated with hyperproliferation, e.g., psoriasis and squamous cell carcinomas. Surprisingly, the expression of these hyperproliferation-associated keratins occurs in the nondividing population of SCC-13 cultures. Moreover, when retinoids are added to the culture medium at a concentration 10-fold higher than physiological, they increase the proliferation of keratinocyte cultures, and yet they inhibit differentiation of both normal and SCC-13 cells. This inhibition includes the suppression of K1/K10 (normal) and K6/K16 (hyperproliferation-associated) keratins. Whether retinoids can reverse the differentiation process in a cell already committed to differentiate terminally or whether retinoids suppress the choice of mitotically active basal cells to undergo a commitment to differentiate terminally awaits additional investigation.

Retinoids are commonly used in the clinical treatment of many different skin disorders. The finding that retinoids can inhibit both the expression of K6/K16 and also the abnormal differentiation typically associated with hyperproliferation might explain some of the therapeutic effects of vitamin A and its analogues on the skin. However, retinoids can increase the population of basal-like cells without a loss in their proliferative potential, which is a worrisome side effect of the successful inhibition of abnormal differentiation.

II. Divergence of Epidermis and Hair Follicle Pathways of Differentiation During Embryogenesis.

All epithelial components of the skin are derived from a single layer of cells, the embryonic basal layer. In the past year this laboratory has begun to elucidate the timing and appearance of biochemical

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differences that lead to distinct programs of differentiation in the epidermis and hair follicle. The embryonic basal layer expresses low levels of the basal epidermal keratins K5 and K14. At this early stage, aggregates of specialized mesenchymal cells, or dermal papillae, form beneath this layer. Where embryonic basal cells come into contact with dermal papillae, they suppress expression of K5 and K14, grow downward, and appear to become committed to form hair follicles. In the absence of this stimulus, embryonic basal cells stratify to give rise to epidermis. K5 and K14 expression is increased in the basal layer and downregulated during terminal differentiation.

As the hair follicle develops, mitotically active matrix cells, which remain in direct contact with dermal papillae and which remain relatively undifferentiated, do not seem to contain keratin filaments, nor do they express K14 and K5. These cells can choose among six different programs of differentiation to give rise to specialized cells of the hair shaft and the inner root sheath. Only upon commitment to one of these programs is expression of hair-specific keratin mRNAs induced. In contrast, the mitotically active cells of the developing outer root sheath do not remain in contact with dermal papillae; concomitant with this loss of interaction, the cells induce expression of K5 and K14, which is maintained in the adult. Further investigation is needed to determine whether there is a specific hair follicle-stimulating factor produced by dermal papillae, whether this factor plays a role in controlling K5/K14 expression, and whether there is a narrow window during development whereby the epithelial component can respond to these cells.

III. Function and Structure of Keratin Filaments.

To form a single 8 nm keratin filament, ~10,000 subunits each of type I and type II keratins interact in a complex self-assembly process. To elucidate the mechanisms underlying this process and to examine the dynamics of the keratin filament network and its interactions with other cellular organelles, the laboratory has focused on deletion and site-directed mutagenesis of the coding portions of the cDNAs encoding human K14. To distinguish the expression of the mutant keratins in epidermal cells, the group replaced the carboxyl-terminal sequence encoding the antigenic portion of K14 with a small sequence encoding the antigenic portion of neuropeptide substance P. An antiserum, anti-K14, recognized only the endogenous K14, while an antibody, anti-P, recognized only the foreign (mutant) K14-P. An SV40 promoter/enhancer was used to drive the expression of these mutant K14-P cDNAs in a variety of cells *in vitro*. Even when sequences encoding the entire nonhelical end domains of K14-P were removed (~150 amino acids), the mutant protein, composed largely of a 310–amino acid residue central helical domain, was able to integrate into the existing keratin filament network. However, when a single point mutation was introduced into a highly conserved region at the end of the α-helical domain, the mutant protein caused complete collapse of the existing keratin filament network. Other mutations showed various phenotypes, and in many cases the phenotype was dominant. The behavior of these mutant keratins in cultured epidermal cells suggested that 1) the keratin filament network is far more dynamic than previously recognized, 2) the network appears to initiate from the nuclear envelope and grow toward the cell periphery, and 3) alterations of the intracellular keratin network in one epidermal cell can influence keratin networks in adjacent cells, presumably indirectly, through desmosomal contacts. *In vitro* filament assembly studies with these mutants should help to unravel the precise nature of keratin subunit interactions. Transgenic mice expressing these mutants should test the possibility that some keratin mutations might lead to various genetic skin diseases.

IV. Regulation of Keratin Gene Expression.

As possible agents for targeting the expression of foreign genes in the skin and for altering the expression patterns of genes normally involved in epidermal differentiation, the promoters/enhancers of the epidermal keratin genes should be invaluable. The laboratory has focused on the human genes encoding K5 and K14, the keratins expressed in the mitotically active cells of the epidermis, and has isolated and characterized the functional genes encoding these keratins. The transcriptional rates of these genes in cultured human basal epidermal cells appear to be the highest of all genes expressed in these cells. The group has begun to elucidate the sequences important in directing the proper tissue-specific and differentiation-specific expression of these genes. Preliminary studies have revealed that sequences encompassing 2,500 bp of a 5′ upstream regulatory sequence of the human K14 gene are sufficient to drive the expression of the tagged K14-P cDNA (see section III) in the basal layer of the epidermis of transgenic mice. Additional studies are in progress to delineate the regulatory elements responsible for this pattern of ex-

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pression and to determine whether similar elements are responsible for determining the program of expression of the human K5 gene.

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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MOLECULAR BASIS OF FERTILIZATION

DAVID L. GARBERS, PH.D., *Investigator*

The major focus of this research continues to be the molecular basis of fertilization, with emphasis on the structures of those molecules that specifically interact with spermatozoan receptors, the identification of the receptor molecules, and the definition of the physiological events that occur in response to receptor occupation. The results obtained with germ cells also provide important information on the mechanisms by which other cells respond to their environment.

I. Primary Structure of the Membrane Form of Guanylate Cyclase.

Initially the mRNA encoding guanylate cyclase from the sea urchin *Arbacia punctulata* was cloned. Crosslinking studies in *Arbacia* had suggested that guanylate cyclase was the receptor for an egg-derived peptide. The cDNA from this sea urchin was used to probe for hybridizing clones in cDNA libraries from another species of sea urchin, as well as from rat and human tissues.

A cDNA clone for the membrane form of guanylate cyclase was isolated from the testis of the sea urchin *Strongylocentrotus purpuratus*. An open reading frame predicted a protein of 1,125 amino acids including an apparent signal peptide of 21 residues. The deduced protein sequence was homologous to the protein kinase family and contained limited but significant regions of identity with a low-molecular-weight atrial natriuretic peptide–clearance (ANP-C) receptor. The carboxyl region (202 amino acids) was 42% identical with a subunit of the cytoplasmic form of guanylate cyclase cloned from bovine lung but was distinctly different from the *Arbacia* sequence in the distal carboxyl region.

The sea urchin clone was successfully used to isolate positive-hybridizing clones from rat brain and human kidney and placental cDNA libraries. The deduced sequences of the human and rat clones were nearly identical. The open reading frame of the rat brain cDNA encoded a protein of 1,057 amino acids, including a predicted 28–amino acid signal peptide.

Further analysis of the deduced amino acid sequence of the guanylate cyclase cDNA indicated that it could be divided into three potential functional domains, based on homology with other proteins. The extracellular domain of the rat membrane guanylate cyclase is 33% identical with the bovine ANP-C receptor, an ANP-binding protein that is apparently not coupled to activation of guanylate cyclase. The ANP-C receptor consists of an extracellular ANP-binding domain, a transmembrane domain, and a short (37 amino acids) cytoplasmic tail. The five cysteine residues of the ANP-C receptor are conserved in guanylate cyclase.

Just within the transmembrane domain an intracellular domain related to the catalytic domain of protein kinases is found, although protein kinase activity has not yet been detected. A 256–amino acid portion of the intracellular domain is 31% identical to the protein tyrosine kinase domain of the platelet-derived growth factor receptor. Guanylate cyclase conforms to the protein kinase consensus sequence in 30 of 33 residues highly conserved or invariant across the protein kinase family. The Gly-X-Gly-X-X-Gly consensus sequence of protein kinases, however, is Gly-X-Gly-X-X-X-Gly in the rat guanylate cyclase.

The highest degree of similarity between the deduced amino acid sequence of the membrane form of guanylate cyclase and other proteins is found in the carboxyl portion of the intracellular domain. A 253–amino acid sequence in this region is 42% identical to the carboxyl terminus of one of the subunits of a bovine soluble form of guanylate cyclase. It is not known whether the sequence determined for the soluble enzyme is from a regulatory or catalytic subunit. In addition, the bovine brain adenylate cyclase contains the two internally homologous domains that are also homologous to this region of guanylate cyclase.

II. Guanylate Cyclase as a Cell-Surface Receptor.

The cloned guanylate cyclase functions as an ANP receptor. Cells transfected with a vector containing the rat brain clone specifically bind ~9 times more ^{125}I-labeled ANP than cells transfected with vector alone. ^{125}I-ANP binds with high affinity and the expected specificity (half-maximal inhibition of binding of 0.4 nM ^{125}I-ANP occurred at ~3 nM unlabeled ANP or 100 nM unlabeled atriopeptin I). To verify that guanylate cyclase acts as an ANP receptor, ^{125}I-ANP was used in crosslinking experiments. Transfected cells were incubated with ^{125}I-ANP, with or without an excess of unlabeled ANP. After crosslinking and SDS-PAGE (reducing conditions), a

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single band migrating with M_r = 130,000 was evident in the cells transfected with the cyclase vector. This band was not seen in the presence of an excess of nonradioactive ANP or in cells transfected with vector alone. The mobility of the radioactive band coincides with that previously reported for the high-molecular-weight ANP receptor. Thus the guanylate cyclase cDNA encodes a protein possessing both guanylate cyclase and ANP-binding activities.

III. Primary Structure of the Resact Precursor.

Previous studies had shown that the speract precursor contained multiple copies of speract and speract-like peptides. In addition, other potential peptides of unknown function were present in the precursor polypeptide. The cDNA encoding resact was isolated and sequenced to determine whether the precursor of an egg peptide from another sea urchin species would contain conserved sequences; this allowed probing for such egg peptides across the species. Unlike the speract precursor, the resact precursor contained only a single peptide corresponding to resact, and there were no significant similarities between the speract and resact precursors.

Dr. Garbers is also Professor of Pharmacology and of Molecular Physiology and Biophysics at the Vanderbilt University School of Medicine.

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MOLECULAR GENETICS OF MEMBRANE AND SECRETORY PROTEINS

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

Investigations in this laboratory focus on the molecular genetics of membrane and secretory proteins. Experiments involve three proteins: 1) the hemagglutinin (HA) of influenza virus, which is being utilized as a marker molecule for specific cell populations in transgenic mouse experiments; 2) human tissue-type plasminogen activator (t-PA), a serine protease that is produced in endothelial cells and is involved in fibrinolysis; and 3) BiP/GRP78, a luminal protein of the endoplasmic reticulum (ER) that appears to be involved in the initial mobilization of proteins that traverse the secretory pathway. This work is undertaken in close collaboration with the laboratory of Dr. Joseph F. Sambrook (University of Texas Southwestern Medical Center at Dallas).

I. Studies on Transgenic Mice Expressing Influenza Hemagglutinin.

Dr. Gething and her colleagues are using transgenic mice to study the development of immunological responses to a well-characterized cell surface antigen. RIPHA mice, which express HA from the rat insulin II promoter/enhancer only in the β-cells of the pancreas, promise to provide a valuable model for the study of immune tolerance and autoimmune diabetes. Additional lines of transgenic mice were recently developed that express wild-type (cell surface) and secreted forms of HA under the control of the mouse metallothionein promoter. These MTHA mice, which express high levels of HA in liver and kidney, will enable investigation of how the immunological response to HA differs when the protein is expressed in major cell populations.

From birth, transgenic RIPHA-33 mice have slightly raised blood glucose levels (190 ± 60 mg/dl) compared with those measured in control animals (136 ± 24 mg/dl). Although histological analysis of the pancreata of young RIPHA-33 mice reveals some disorganization of the normally ordered architecture of the islets, these animals display no physiological problems until ~4–5 months of age. At this time, increases in the blood glucose levels of individual mice to >300 mg/dl begin to be observed, and shortly afterward these mice develop hyperglycemia (blood glucose >400–650 mg/dl), which is responsive to administration of insulin. Such changes in blood sugar levels are not observed in control animals. By 9 months a significant fraction of the RIPHA-33 mice (60% of the males, 10% of the females) have developed frank diabetes mellitus. Histological analysis of the pancreata of such animals reveals many islets with disrupted morphology, as well as destruction of β-cells and evidence of insulitis. The majority of the invading immune cells stain with anti-CD4 antibodies, suggesting that they are helper/DTH cells; others stain with anti-CD8 antibodies and may be cytotoxic T cells. Analyses of the sera of diabetic animals often reveal the presence of anti-HA antibodies, as well as antibodies against islet cell antigens.

II. Structure-Function Studies on Tissue-Type Plasminogen Activator.

The biological function of t-PA is to convert the inactive zymogen, plasminogen, into the active protease plasmin. In the circulation the level of t-PA activity is controlled by the interaction of the molecule with three other proteins. First, the affinity of the enzyme for its substrate plasminogen is increased several hundredfold by binding to fibrin. Second, t-PA is rapidly inactivated by the serpin plasminogen activator inhibitor-1 (PAI-1). PAI-1 acts as a suicide substrate and forms a covalent bond with Ser-478 in the active site of t-PA. Finally, the enzyme is efficiently cleared from the circulation by specific t-PA receptor(s) on hepatic cells.

Synthesized and secreted as a single polypeptide chain, t-PA is subsequently cleaved into two subunits held together by a single disulfide bond. The carboxyl-terminal light chain constitutes the catalytic domain of the molecule and shares homology with other members of the serine protease family. The heavy chain is composed of a number of independent structural domains that are encoded by individual exons in the t-PA gene. These include 1) a "finger" domain having homology to the fibrin-binding finger domains of fibronectin, 2) an epidermal growth factor (EGF)-like domain, and 3) two "kringle" structures having homology to similar domains found in numerous other serum proteins. A set of mutant enzymes lacking individual structural domains of the heavy chain was generated to determine which domains of t-PA interact with the various effector molecules. Although the finger and EGF-like domains are involved in the initial, high-affinity binding of t-PA to fibrin, stimulation of t-PA ac-

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tivity requires secondary, lower affinity interactions of fibrin with either of the two kringle domains. The binding of the t-PA molecule to specific receptors on hepatic cells also involves sequences within the finger and/or EGF-like domains. Finally, the absence of heavy-chain domains has no effect on the interaction of PAI-1 with the catalytic light chain.

Although the three-dimensional structure of t-PA has not been elucidated, Dr. Gething and her colleagues have been able to model the EGF-like domain, the kringle domains, and the light-chain/inhibitor complex, using the known structures of homologous proteins. Site-directed mutants designed using these proposed structures have provided information about the individual amino acid sequences that interact with the effector molecules. For example, the group has generated variant enzymes that are efficient, fibrin-stimulated plasminogen activators but 1) are resistant to inhibition by a variety of serpins, including PAI-1, or 2) 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.

III. Endoplasmic Reticulum Proteins Involved in Folding and Mobilization of Nascent Polypeptides.

Prefolded or malfolded forms of HA bind to a 77 kDa cellular protein present in the lumen of the ER. A second ER protein (94 kDa) associated with nascent, unglycosylated HA synthesized in the presence of drugs that inhibit the addition of amino-linked oligosaccharides to the newly synthesized polypeptide. As part of the effort to understand the role of protein folding in intracellular transport, biochemical and genetic techniques are being used to characterize the structure and function of these two ER proteins.

A full-length cDNA encoding the 77 kDa protein has been cloned and expressed from murine cells. On the basis of amino acid sequence, immunological reactivity, and functional activity, it has been established that this protein corresponds to two previously described ER proteins, the immunoglobulin heavy-chain binding protein (BiP) and the glucose-regulated protein (GRP78), and that it is a member of the HSP70 multigene family, which includes the cytoplasmic 70 kDa heat-shock proteins. A full-length cDNA encoding the murine 94 kDa ER protein has also been cloned. Like GRP78, GRP94 is related to cytoplasmic heat-shock proteins, displaying 45% sequence identity with HSP90. These glucose-regulated proteins, which are major constituents of the ER of mammalian cells, are synthesized constitutively under normal growth conditions but are induced under a variety of conditions of stress, with the common denominator the accumulation of unfolded polypeptides in the ER.

The gene encoding BiP from the yeast *Saccharomyces cerevisiae* has been cloned, using the murine BiP cDNA as a hybridization probe. This gene is essential for viability of yeast cells. Surprisingly, the coding sequence of yeast BiP is identical to that of the *KAR2* gene, one of a class of genes involved in nuclear fusion after mating of yeast cells. Expression of mammalian BiP in *S. cerevisiae* can complement a mutant allele of *KAR2* that is temperature sensitive for growth and nonconditionally defective for karyogamy.

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 CELLULAR METABOLISM AND FUNCTION

JOHN A. GLOMSET, M.D., *Investigator*

Two major areas of investigation are being pursued in Dr. Glomset's laboratory: 1) the structure, metabolism, and function of membrane lipids and 2) the structure and function of membrane proteins that are modified by isoprene groups.

I. Structure, Metabolism, and Function of Membrane Lipids.

In collaboration with Dr. Andreas Habenicht (University of Heidelberg), mechanisms that control the formation of prostaglandins and other eicosanoids are being studied in replicating fibroblasts and differentiating macrophages. Early experiments with replicating Swiss 3T3 cells in culture showed that these cells produce prostaglandin E_2 in two phases during the cell cycle. A transient, early phase occurs almost immediately in response to the addition of platelet-derived growth factor (PDGF), whereas a second phase occurs after the cells have traversed the cell cycle for 2–6 h. The first phase resembles that seen in many other prostaglandin-response systems, in that the arachidonic acid that is required for prostaglandin production is released from membrane phospholipids by one or more phospholipases. In contrast, the second phase of prostaglandin formation only occurs in the presence of exogenous unesterified arachidonic acid or low-density lipoproteins (LDL) and is associated with the PDGF-dependent upregulation of at least two major enzymes of prostaglandin synthesis, prostaglandin H synthase and prostacyclin synthase.

Experiments conducted during the past year provided important new information about the mechanism of the LDL effect. The effect is clearly mediated by the LDL receptor, which also is upregulated in response to PDGF. Thus only low concentrations of LDL are required, antibodies to the LDL receptor block the effect, and the effect is not seen in fibroblasts from patients with familial hypercholesterolemia. Uptake and degradation of LDL seem to be required, because the effect of LDL is blocked by chloroquine, an inhibitor of lysosomal hydrolases. Furthermore, experiments with reconstituted LDL containing labeled, esterified arachidonic acid have shown that the effect of LDL depends on the delivery of arachidonic acid for prostaglandin production. This is a new role for the LDL receptor that might be of considerable regulatory importance. Experiments are under way to explore its significance for cell cycle progression.

II. Isoprene-containing Proteins.

Experiments in Dr. Glomset's laboratory, reported last year, demonstrated that lamin B, a structural protein associated with the nuclear envelope, is modified by an isoprene group. Recent experiments, in collaboration with Dr. Michael Gelb (University of Washington), have provided evidence that a cysteine residue at the carboxyl end of lamin B contains a thioether-linked farnesyl group. This is the first direct evidence of the farnesylation of animal cell proteins.

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

POLYPEPTIDE HORMONE GENE REGULATION

JOEL F. HABENER, M.D., *Investigator*

Major emphasis in Dr. Habener's laboratory is presently in two areas: 1) identification 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 either to up- or downregulate the transcription of genes. A major goal is to isolate and characterize the structures and functions of the DNA-binding proteins. Dr. Habener is investigating the cell-specific expression of the glucagon, somatostatin, and angiotensinogen genes, using islet cell lines with distinct hormone-expressing phenotypes. 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 analyses of the expression of the human glycoprotein hormone α-subunit gene, utilizing the JEG-3 placental cell line, in which the transcription of the α gene is greatly stimulated by cAMP. The transcription of the α and CG-β genes is stimulated by analogues of cAMP, and synthetic cAMP/enhancer-like cassettes linked to the α-promoter in bacterial chloramphenicol acetyl transferase (CAT) reporters confer a 40- to 50-fold induction of gene transcription. Dr. Habener is investigating the molecular workings and interactions of the multiple cooperating cis elements and DNA-binding proteins involved in cellular cAMP responsivity at the level of the genome.

One cis element of the α gene consists of two direct 18 bp repeats, each of which contains the palindromic cAMP-responsive octamer motif, TGACGTCA (CRE). This sequence element confers cAMP responsiveness and enhancer-like properties to the gonadotropin α-subunit, glucagon, and somatostatin genes.

The structural and functional properties of the CRE were investigated. Dr. Habener found that the CRE only functions as a transcriptional response element when present in the proper context of adjacent bases surrounding the elements. The cAMP responsiveness of the CRE depends on the catalytic subunit of cAMP-dependent protein kinase A, because all responsiveness was either stimulated or attenuated by coexpression of minigenes encoding either the catalytic subunit of protein kinase A or the protein kinase A inhibitor peptide, respectively.

Dr. Habener determined the amino acid sequence of CREB from a cloned cDNA that he isolated from a placental λgt11 expression library using a radioactive CRE probe. CREB is a 327–amino acid protein that belongs to a newly recognized class of transcriptional proteins, the "leucine zipper" proteins. Other members of this class of proteins include myc, fos, c-jun, C/EBP, and GCN4. The zipper, located at the carboxyl terminus of CREB, consists of a heptad repeat of leucine residues interspersed with charged residues that form an amphipathic α-helix with a hydrophobic face, allowing two CREB proteins to interact to form a parallel coiled-coil CREB homodimer. A positively charged sequence with ~40% amino acid identities with jun and fos (basic region) lies adjacent to the leucine zipper region. Dr. Habener proposes that the carboxyl-terminal basic region juxtaposed to the zipper region forms a helix-turn-helix with two amphipathic α-helices with basic faces, which constitutes the DNA-binding domain of CREB that binds to the symmetrical CRE palindromes as a homodimer. A synthetic peptide consisting of the carboxyl-terminal 66 residues of CREB readily forms dimers and binds the CRE, but a peptide of 50 residues, missing one helix of the basic region, forms dimers but does not bind the CRE. Furthermore, cell-free cotranslation of CREB, jun, and fos in different combinations shows the formation of CREB homodimers and jun/fos heterodimers but no CREB/jun or CREB/fos heterodimers, indicating that the CREB/CREB dimer is a highly favored configuration. The amino-terminal region of CREB is negatively charged and exists in the conformation of a random coil—characteristic of a "negative noodle" believed to be involved in transcriptional activation. In addition, this random coil region of CREB contains a sequence of 50 residues containing phosphorylation sites for protein kinases A and C, casein kinase II, and glycogen synthase kinase III. This phosphorylation box (P-box) sequence has the potential for forming an amphipathic α-helix with an acid face when the serines are converted to phosphoserines. Dr. Habener has shown that the P box is responsible for transcriptional activation in response to cAMP, because fusion genes consisting of the CREB sequence 1–260 (negative noodle), both with and without the P box, linked to the

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GAL-4 DNA-binding domain, activate transcription of a UAS reporter only when the P box is present. The P-box sequence of 50 residues alone is sufficient to transactivate transcription. Analyses of both mRNAs and genomic DNA fragments indicate a considerable complexity of CREB and CREB-like products. Curiously, several of the mRNAs and gene fragments hybridize to both CREB and jun cDNAs, although these two cDNAs do not cross-hybridize with each other.

During the next year, research will be focused on more-detailed investigations of the functional domains of the cAMP-responsive DNA-binding protein. Studies will be aimed at defining the DNA-binding properties and the molecular processes of transcriptional activation. Emphasis will be on investigations of the roles of phosphorylation and glycosylation in nuclear transport, DNA binding, dimerization, and coupling to other transcription factors. The structure of the CREB gene and the diversity of additional CREB-like gene products in other tissues will be investigated. Efforts will be made to isolate the cDNAs encoding the upstream and downstream DNA-binding proteins that cooperatively enhance cAMP-mediated activation of transcription of the gonadotropin α and somatostatin genes.

Dr. Habener has also examined 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 also two additional peptides related in structure to glucagon, termed glucagon-like peptides. Marked differences in the pattern of post-translational processing of glucagon were found in rat pancreas, which produces glucagon, and intestine, which produces predominantly glucagon-like peptides.

Having established that proglucagon encodes new glucagon-like peptides, Dr. Habener investigated the potential biologic activities of these new peptides. He discovered that 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 is distinct from that of glucagon in hepatic cells. Administration of the synthetic peptide to human volunteers results in a marked increase of plasma insulin levels followed by a fall in blood glucose levels. Dr. Habener is planning to test the possible therapeutic properties of GPL-I in patients with non-insulin-dependent diabetes mellitus.

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

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

NATHANIEL HEINTZ, PH.D., *Associate Investigator*

The studies pursued in Dr. Heintz's laboratory are guided by the thought that most interesting biological transitions, whether within the life of a single cell or during the development of a complex tissue, are accompanied by changes in the expression of specific genes. Thus knowledge of the molecular events that result in the activation of these genes in response to such a transition can lead to a detailed understanding of the transition. Dr. Heintz is utilizing this approach to examine specific transitions that occur in two different biological contexts: the mammalian cell division cycle and the developing mouse cerebellum.

I. Control of Gene Expression During the Cell Cycle.

Approximately 20 years ago it was demonstrated that histones are synthesized at significant rates only during the S phase of the cell cycle. Early studies in this and other laboratories established that these proteins are encoded by a small supergene family and that transcriptional control is important for the increased expression of these proteins during the transition from G1 to S phase. Furthermore, Dr. Heintz and his colleagues demonstrated that transcriptional regulation of histone gene expression is due to subtype-specific transcription factors that bind to highly conserved sequence elements shared by individual genes coding for a particular histone subtype and that this type of regulation could be reproduced *in vitro*. These observations led to a simple model for coordinate induction of histone gene expression, involving activation of distinct transcription factors by a common mechanism that becomes active during the transition from G1 to S phase.

During the past year, Dr. Heintz's laboratory has continued analysis of five histone gene transcription factors (H4TF1, H4TF2, OTF1, H1TF1, H1TF2) and their role in cell cycle regulation of transcription. In particular, in-depth analysis of histone H1 transcription in extracts from homogenous populations of G1 and S phase cells prepared by centrifugal elutriation has confirmed and extended the model that histone gene regulation is mediated by subtype-specific transcription factors. In this case, S phase induction is achieved through the agency of two distinct H1-specific transcription factors, H1TF1 and H1TF2. Quantitative DNA-binding assays indicate that, in contrast to H1TF1 and the H2b factor OTF1, H1TF2 DNA binding is elevated in S phase HeLa cells. Thus, although all three of these factors directly participate in coordinate activation of histone gene expression upon entry into S phase, their biochemical response to this transition is not uniform. Current efforts focus on chemical characterization of each of the histone-specific transcription factors and generation of monospecific antibodies to them. Relatively large quantities of H4TF2, OTF1, H1TF1, and H1TF2 have been purified and are being utilized for these purposes.

Dr. Heintz's laboratory has also begun to investigate whether the same mechanisms alluded to above might regulate other cellular genes whose expression is temporally controlled during the cell cycle. In particular, experiments have been initiated to dissect the human thymidine kinase (TK) promoter (in collaboration with Dr. S. Conrad) to identify DNA sequences and protein factors important for their temporal regulation. Several novel factors interacting with the TK promoter have been identified, although a role for these proteins in cell cycle regulation has not been established. The observation that both of the histone H1 cell cycle regulatory factors specifically interact with the TK promoter suggests a possible common mechanism for regulation of TK and H1 gene expression.

II. Control of Gene Expression in 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 the isolation of genes that are either required for or respond to specific transitions that occur during the development of the mouse cerebellum. To identify genes that are required for

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normal cerebellar function, phenotypic crosses of the *lurcher* (*lc*) and *meander tail* (*mea*) neurological mutants with *Mus castaneous* have been used to map these genetic defects on mouse chromosomes 6 and 4, respectively. The well-studied semidominant mutation *lc* results in degeneration and death of essentially all cerebellar Purkinje cells, commencing at about postnatal day 10. The fully recessive mutation *mea* results in skeletal abnormalities in the tail, as well as cerebellar abnormalities. Since no detailed analysis of the *mea* phenotype has yet been published, Dr. Heintz's laboratory (in collaboration with Drs. Carol Mason and Mary Beth Hatten, Columbia University College of Physicians and Surgeons) has initiated an in-depth study of cerebellar anatomy in the *mea* mouse. The adult *mea* homozygote displays normal foliation and cytoarchitecture in the posterior lobes of the cerebellum. However, the anterior lobes of the *mea* cerebellum are characterized by the gross disorganization of the Purkinje cells, the absence of granule cells, and disorientation of radial glia. The facts that the transition from normal to grossly abnormal cytoarchitecture occurs very rapidly in the *mea* cerebellum, that the transition occurs in the same position in different animals, and that this line of demarcation is present as early as postnatal day 8 suggest that the *mea* gene product defines a specific developmental compartment in the mammalian brain. Present efforts are aimed at saturating the genetic map surrounding these neurological phenotypes with anonymous DNA probes and constructing long-range physical maps in the appropriate genetic interval, in preparation for cloning the *lc* and *mea* genes.

A second cloning effort, which involves the use of subtractive cloning and plus/minus screening of libraries prepared from RNA isolated from either wild-type or mutant animals is under way. The object of this work is to isolate a wide variety of genes that are expressed either in specific cell types or with a specific developmental profile in the mouse cerebellum.

Dr. Heintz is also Associate Professor and University Fellow at The Rockefeller University.

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MOLECULAR ANALYSES OF CELL-MATRIX ADHESION

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

The laboratory is 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 of which are 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 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. For example, two of the segments (A and B) are always present in the fibronectin associated with cell migration during development. They 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 fibronectin 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 hypothesis and others based on the descriptive studies of the expression and splicing patterns of different fibronectin isoforms, recombinant fibronectin genes were constructed and introduced into cells to produce cell lines that secrete in pure, homogeneous form each of the forms of fibronectin that, in nature, are found in mixtures. In this way it is possible to purify the different forms in quantity and to assay their biological functions. One result of these studies is that certain lymphoid cells adhere specifically only to those forms of fibronectin that contain 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 ($\alpha_4\beta_1$) that recognizes this site was identified by affinity chromatography on synthetic peptides and by specific antibody blocking.

Studies are under way to analyze further the roles of the three alternatively spliced segments, both *in vitro* and *in vivo*. Projects have been initiated to prepare transgenic mice expressing specific forms of fibronectin inappropriately and to "knock out" the fibronectin gene in embryonic stem (ES) cells in order to generate mice with mutant fibronectin genes. Finally, using DNA hybridization and polymerase chain reaction, attempts are in progress to identify and clone the gene for fibronectin in *Drosophila*. Success in this latter project would open the way to the application of the sophisticated genetic manipulations possible in this organism.

Integrins are transmembrane receptors made up of α- and β-subunits. There are at least 6 β-subunits and at least 11 α-subunits. Different αβ combinations generate receptors with different but overlapping specificities for various adhesive extracellular matrix proteins. 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. This model is under test by transfection experiments.

The roles of integrins in development are being studied genetically in *Drosophila*. The gene for a *Drosophila* β-integrin has been cloned, and mutants are available. Gynandromorphs and somatic clones reveal specific defects in patches where integrins are deleted. Reintroduction of wild-type and mutant integrins into the mutant strains will allow further analysis of the roles of these proteins.

The nature of the cytoskeletal connection is being examined by transfection of mutated integrin subunit genes into 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, with particular attention to three related integrin receptors ($\alpha_3\beta_1$,

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$\alpha_4\beta_1$, $\alpha_5\beta_1$). These integrin receptors are all receptors for fibronectin, albeit for different regions, including, in the case of $\alpha_4\beta_1$, 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 now being used to determine the binding sites for potential interacting proteins, including integrins, vinculin, and calpain. The aim is to elucidate the molecular structure of the "focal contact" where extracellular matrix is connected to the cytoskeleton via integrins. This is an important point of regulation of cell behavior.

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

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PROTEIN PHOSPHORYLATION AND INTRACELLULAR SIGNALING

EDWIN G. KREBS, M.D., *Senior Investigator*

The primary focus of this laboratory has been elucidation of the role of protein phosphorylation reactions in signal transduction. To this end the regulatory properties of several messenger-dependent and messenger-independent protein serine kinases have been investigated. In addition, limited studies have been carried out on protein serine phosphatases. A new area of interest is a heretofore neglected group of enzymes, the protein tyrosine phosphatases.

I. Activation of Protein Kinases in Amphibian and Echinoderm Oocytes.

In experiments directed toward examining the role of protein phosphorylation in the meiotic cell cycle, a synthetic peptide, Arg-Arg-Leu-Ser-Leu-Arg-Ala (the structure of which is based on a phosphorylated sequence in ribosomal protein S6), was employed as a probe for measuring protein serine kinase activity in *Xenopus laevis* oocytes induced to mature by insulin or progesterone. Insulin elicited an early (20–30 min) 3-fold stimulation of S6 peptide-phosphorylating activity that was not evident with progesterone. However, both hormones produced a delayed 7- to 12-fold stimulation of S6 peptide-phosphorylating activity at the time of germinal vesicle breakdown (GVBD). The results of DEAE-Sephacel, Sephacryl S-200, TSK-400, and heparin-Sepharose chromatographic fractionation experiments implied that a common S6 peptide kinase was activated as a consequence of short- and long-term insulin exposure, as well as in long-term progesterone treatment of oocytes. Further work on the pathways involved in the activation of the S6 peptide kinase is being undertaken.

In addition to examining protein kinase activation in amphibian oocytes, studies were also undertaken using starfish (sea star) oocytes, which are abundant in Puget Sound and readily available to this laboratory. (In this organism, 1-methyladenine is the natural hormone that induces maturation, instead of progesterone, as in vertebrates.) Five activated protein kinases were detectable in soluble extracts from maturing, as compared with immature, sea star oocytes. These kinases could be distinguished on the basis of the time courses of their activation after exposure of the oocytes to 1-methyladenine, their substrate specificities, and their chromatographic properties. A histone H1 kinase (HH1K; M_r 110,000) underwent maximal activation near the time of 1-methyladenine–induced GVBD. When myelin basic protein (MBP) was used as a substrate, HH1K and two additional kinases (MBPK-I and MBPK-II) were detectable. MBPK-II (M_r 110,000) was fully activated at the time of GVBD, whereas peak activation of MBPK-I (M_r 45,000) occurred after this event. Two "ribosomal protein kinases" (S6K-I and S6K-II) underwent activation post-GVBD. The HH1K and MBPK-II activities both declined prior to the emission of the first polar body (i.e., meiotic cell division), but the MBPK-I, S6K-I, and S6K-II activities remained elevated during this time. Developments in other laboratories during the past year make it seem probable that HHIK is a component of the maturation-promoting factor (MPF).

II. Casein Kinase II.

This laboratory has a special interest in a messenger-independent protein kinase known as casein kinase II (CK-II), which is activated when cells are exposed to growth factors. CK-II is found in the nucleus, as well as in the cytoplasm, and in this connection it is of considerable interest to find that the kinase catalyzes the phosphorylation of a number of nuclear oncoproteins. It was shown, for example, that cellular *myc*-encoded proteins can serve as substrates for phosphorylation by purified CK-II *in vitro* and that this phosphorylation is reversible. One- and two-dimensional mapping experiments demonstrated that the major phosphopeptides from *in vivo* phosphorylated Myc corresponded to the phosphopeptides produced from Myc phosphorylated *in vitro* by CK-II. In addition, synthetic peptides with sequences corresponding to putative CK-II phosphorylation sites in Myc were subject to multiple, highly efficient phosphorylations by CK-II and could act as competitive inhibitors of CK-II phosphorylation of Myc *in vitro*. It was postulated that CK-II–mediated phosphorylation of Myc plays a role in signal transduction to the nucleus.

III. Activation of Protein Serine Phosphatase 1 by Growth Factors.

Although most workers have concentrated on protein kinases as the targets for regulation by

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growth factors, it was determined in this laboratory that protein phosphatases are also subject to regulation. Incubation of Swiss 3T3-D1 cells with physiological concentrations of insulin resulted in a rapid and transient activation of protein phosphatase activity. Activation reached a maximum level (140% of control value) within 5 min of addition and returned to control levels within 20 min. This activity could be completely inhibited by addition of the heat-stable protein inhibitor 2, which suggests the presence of an activated type-1 phosphatase. Similar effects on phosphatase activity were seen when epidermal growth factor and platelet-derived growth factor were tested.

IV. Protein Tyrosine Phosphatases and Signal Transduction.

With the finding that the receptors for a number of growth factors are protein tyrosine kinases and that a number of oncogenes encode similar enzymes, attention has been focused on tyrosine phosphorylation-dephosphorylation as a mechanism involved in intracellular signaling. Until recently almost all of the work in this area involved the tyrosine kinases, but during this past year some of the emphasis has shifted to studies of the protein tyrosine phosphatases (PTPases).

In collaboration with Dr. E. H. Fischer's group, this laboratory has been studying several PTPases, one of which, PTPase 1B, was obtained from human placenta in the pure form. It was found to consist of a single chain of 321 residues with an *N*-acetylated amino-terminal methionine and an unusually proline-rich carboxyl-terminal region. The enzyme is related structurally to the two cytoplasmic domains of both the leukocyte common antigen CD45 and LAR, a CD45-like molecule with an external segment that resembles a neural cell adhesion molecule. A low-molecular-weight protein encoded by a cDNA clone from T cells (see below) also shows extensive sequence similarities. Homologous domains common to this diverse family of PTPases were defined.

A human peripheral T cell cDNA library was screened with two labeled synthetic oligonucleotides encoding regions of the human placental PTPase 1B. One positive clone was isolated, and the nucleotide sequence was determined. It contained 1,305 base pairs of open reading frame followed by a TAA stop codon and 978 base pairs of 3′ sequence. An initiator methionine residue was predicted at position 61, which would result in a protein of 415 amino acid residues (M_r 48,400). This was supported by the synthesis of an M_r 48,000 protein in an *in vitro* reticulocyte lysate translation system using RNA transcribed from the cloned cDNA and T7 RNA polymerase. The deduced amino acid sequence was compared with other known proteins, revealing 65% identity to the low-molecular-weight PTPase 1B isolated from placenta. In view of the high degree of similarity, the T cell cDNA likely encodes a newly discovered protein tyrosine phosphatase, thus expanding this family of genes. Experiments have been undertaken to overexpress this enzyme in transformed cells.

Dr. Krebs is also Professor of Pharmacology and of Biochemistry at the University of Washington School of Medicine.

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MICROBIAL AND MAMMALIAN SULFUR METABOLISM

NICHOLAS M. KREDICH, M.D., *Investigator*

I. Regulation of Cysteine Biosynthesis in *Salmonella typhimurium*.

A. *Interactions of CysB protein with the* cysJIH *promoter.* CysB protein is a tetramer of identical 36 kDa subunits that serves as a transcriptional activator for expression of the cysteine regulon, i.e., the genes required for *de novo* cysteine synthesis in *Escherichia coli* and *Salmonella typhimurium*. Transcriptional activation also requires acetylserine, which in its role as a signal of sulfur deprivation acts as an internal inducer for the cysteine regulon. *In vitro* studies in Dr. Kredich's laboratory have shown that CysB protein binds to a portion of the *cysJIH* promoter, designated CBS-J1, that extends from positions −80 to −35 relative to the transcription start site. Acetylserine stimulates binding three- to eightfold and is required for bound CysB protein to form a transcription initiation complex with RNA polymerase. These results show that the effects of CysB protein are similar to those of other positive regulatory proteins, where an interaction with RNA polymerase is postulated to occur just upstream of the RNA polymerase-binding site. Dr. Kredich and his associates have also examined the effects of sulfide and cysteine on *in vitro* interactions between purified CysB protein and the *cysJIH* promoter. *In vivo* these compounds interfere with the ability of inducer to stimulate expression of genes of the cysteine regulon. *In vitro* studies indicate that sulfide is an anti-inducer, which competes with the stimulatory effects of acetylserine on CysB protein binding and on transcription initiation. Cysteine had no measurable effects *in vitro*, and its *in vivo* effects are postulated to be secondary to conversion to sulfide via cysteine desulfhydrase.

B. cysB *autoregulation*. CysB protein is encoded by the *cysB* gene. The transcription initiation start site for *cysB* has been identified by primer extension studies, and *in vitro* DNA-binding and DNase I protection experiments show that CysB protein binds to the −10 region of the *cysB* promoter at a site designated CBS-B1. In transcription run-off experiments with *cysB*, CysB protein has been shown to prevent formation of a transcription initiation complex, presumably by binding to the promoter and blocking access to RNA polymerase. In contrast to the results obtained with the CBS-J1 site of the *cysJIH* promoter, the inducer acetylserine inhibits binding of CysB protein to CBS-B1 and restores transcription initiation at the *cysB* promoter. These findings corroborate and expand earlier *in vivo* studies with *cysB-lac* fusions, which indicated that *cysB* is autoregulated.

C. *Interactions of CysB protein with the* cysK *promoter.* Dr. Kredich's laboratory has found that there are two contiguous CysB protein–binding sites in the *cysK* promoter region. The first, designated CBS-K1, extends from position −78 to −39 relative to the major transcription start site; the second, designated CBS-K2, extends from position −123 to −87. CysB protein binds readily to both sites in the absence of inducer, but inducer stimulates binding to CBS-K1 while inhibiting binding to CBS-K2. In this regard, CBS-K1 resembles CBS-J1, and CBS-K2 behaves more like CBS-B1. Removal of CBS-K2 by site-directed mutagenesis eliminates CysB protein binding to this region but does not affect binding to CBS-K1 or *cysK* promoter function, as assessed by *in vivo* expression and by *in vitro* transcription run-off assays. Upstream deletions extending into CBS-K1 and certain point mutations in CBS-K1 diminish or totally abolish *cysK* promoter activity both *in vivo* and *in vitro*. Thus CBS-K1 has been identified as the site required for the positive regulatory effect of CysB protein and inducer. Unlike the situation with CBS-B1, there is no evidence that binding to CBS-K2 inhibits *cysK* promoter activity, and the significance of this site is unknown.

D. *Catalytic mechanism of sulfite reductase hemoprotein.* A large number of mutations have been introduced into the *E. coli* sulfite reductase hemoprotein in a collaborative study with Dr. Lewis Siegel (Duke University), which is designed to characterize amino acid residues involved in electron transfer between the Fe_4S_4 and siroheme prosthetic groups of this protein. In attempting to construct a high-level expression vector for large-scale purification of these mutant proteins, researchers in Dr. Kredich's laboratory have discovered that the cofactor siroheme is a limiting constituent for holoenzyme production and that siroheme-deficient apoenzyme is toxic to cells. This obstacle has been overcome recently by constructing a plasmid that contains both the structural gene for the apoprotein (*cysI*) and the gene for the enzyme catalyzing the final step in siroheme synthesis (*cysG*). The cur-

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rent construct now gives holoenzyme levels of 3–4% of total protein. As a part of this project, the *cysG* gene has been identified and sequenced.

II. Regulation of *S*-Adenosylmethionine Metabolism.

Previously reported studies from Dr. Kredich's laboratory showed that human lymphocyte *S*-adenosylmethionine synthetase is composed of nonidentical subunits. Immunologic and enzymatic studies now indicate that the α-subunit is catalytic and that the function of the β-subunit may be regulatory. Attempts are now under way to clone the genes for both subunits as part of an overall effort to characterize the different roles of these subunits and their expression in different cell types. cDNA for the hepatic enzyme from rat has already been cloned and sequenced in Dr. Kredich's laboratory. Comparison of its sequence to those of the *E. coli* and yeast enzymes reveals a high degree of identity. Comparison of the primary structures of the hepatic and lymphocyte enzymes will help explain the marked kinetic difference between these enzymes.

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

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MECHANISMS OF THYROID HORMONE ACTION

P. REED LARSEN, M.D., *Investigator*

Dr. Larsen's laboratory is investigating the mechanism of thyroid hormone action. He has focused on two steps in this process: 1) the deiodination of the prohormone thyroxine (T_4) to produce the active hormone 3,5,3′-triiodothyronine (T_3) and 2) the mechanism by which T_3 alters the expression of the thyroid hormone–dependent protein, rat growth hormone (rGH).

The deiodination of T_4 to produce the active hormone T_3 is a complex process. Several goals must be achieved. Circulating T_3 for use by muscles, heart, liver, and kidney must be produced. A local source of T_3 for the brain must be provided, since circulating T_3, which does not enter this tissue efficiently, is required for normal central nervous system development. Finally, both the circulating active hormone, T_3, as well as the prohormone, T_4, must be monitored. Dr. Larsen's previous work elucidated the relative roles of the two 5′-iodothyronine deiodinases that subserve these goals. One enzyme, the type I deiodinase, is present at highest concentration in the liver and kidney and provides most of the circulating T_3. Type II deiodinase produces ~80% of the specifically bound intranuclear T_3 for the brain and ~50% of that found in nuclei of pituitary and brown adipose tissue. In the pituitary, type II deiodinase permits the thyrotroph to sense the circulating T_4 concentration; in the brown adipose tissue, the T_3 generated by adrenergic stimulation of the type II deiodinase enzyme is required for a normal thermogenic response to cold stress. Type II deiodinase can provide a significant fraction of the body's circulating T_3 under special circumstances (e.g., in the neonatal or hypothyroid rat), since its activity is increased and that of type I deiodinase is reduced.

A major goal of Dr. Larsen's studies has been to identify these membrane-bound deiodinase proteins, learn how they are regulated in different tissues, and analyze their structural-functional relationships. Because of the 20- to 30-fold increases in type II deiodinase activity induced by sympathetic stimulation in brown fat, dispersed brown adipocytes were employed to evaluate its regulation. In earlier studies, catecholamines were found to stimulate type II deiodinase activity, and the effect of the dual agonist norepinephrine (NE) was enhanced by β-adrenergic blockade in cells from euthyroid rats. This emphasized the important role of α_1-agonists in the control of this enzyme. NE stimulation of type II deiodinase was greater in hypothyroid rats but was inhibited by β-blockade. The recent studies showed that both β-adrenergic agents (through cAMP) and α_1-agonists (probably via increasing intracellular calcium) were required for deiodinase stimulation. In both euthyroid and hypothyroid cells, the response to these agents was synergistic. For example, in hypothyroid cells the increment with combined α_1/β-stimulation was four times that which could be explained on the basis of the additive effects of the two pathways. Maximal stimulation required a concomitant exposure of ~1.5 h to both α_1- and β-agonists. It was blocked by actinomycin D and thus required the synthesis of a critical protein, either the enzyme or a species that can activate it. The cAMP response to β-adrenergic agents was reduced in hypothyroid cells, although this became apparent only over a 2 h incubation. In euthyroid cells the magnitude of the deiodinase response to Bt_2 cAMP or forskolin alone was biphasic, with lower stimulation occurring at higher cAMP concentrations. This explained the observation that β-blockade enhanced the response of such cells to NE. Thus the attenuation of the cAMP response in hypothyroid cells was coupled with a greater stimulation in the presence of α_1-agonists. These results are the first demonstration of a compensatory mechanism by which hypothyroidism enhances a cAMP-dependent process. The importance of facultative thermogenesis to survival in the cold points to a teleological rationale for this phenomenon, but the actual mechanism for the increase in type II deiodinase under the α/β-adrenergic influence remains to be determined. It will require the identification of the type II deiodinase or its mRNA.

Although Dr. Larsen's group has obtained several hundred–fold purification of this enzyme using combinations of hydrophobic interaction chromatography and ion-exchange techniques, several bands are still present on silver stains. Therefore they decided to pursue an alternate strategy to isolate these deiodinases. Dr. Marla Berry has employed the *Xenopus* oocyte expression system. The oocyte expresses type I deiodinase activity after injection of poly(A)$^+$ mRNA isolated from hyperthyroid rat liver. Size fractionation shows the mRNA to be ~1.9–3 kb in length. Preliminary results are encouraging, in that deiodinase activity was found after injection of mRNA generated from a pool of 20,000 λ-phage cDNA clones prepared from hyper-

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thyroid liver mRNA. This pool is now being subdivided to identify the cDNA that codes for the functional type I deiodinase enzyme.

The other major focus of Dr. Larsen's activities has been the study of T_3 regulation of gene expression. This project has involved Dr. Ronald Koenig (University of Michigan) and Dr. Gregory Brent. It has been performed in collaboration with Dr. David Moore (Massachusetts General Hospital).

To identify the sequences in rGH that are critical for T_3 stimulation, a functional assay has been used in which rGH promoter/chloramphenicol acetyltransferase (CAT) constructs are transfected into pituitary tumor cells. Dr. Larsen's previous studies had demonstrated the necessity for sequences between –189 and –172 in the rGH promoter to confer complete T_3 responsiveness. The sensitivity and precision of this analysis were limited by the only two- to threefold T_3 induction of the constructs containing rGH T_3RE (T_3 response element). However, cotransfection of a β T_3 receptor–expressing plasmid with the rGH constructs led to a sixfold amplification of the T_3 response. This demonstrated that the T_3 receptor is rate limiting in these GH4C1 cells. The greater induction allowed the recognition of the importance of an additional sequence between –172 and –167, relative to the start site, which also contributed to the T_3 response. A single G to T mutation at nucleotide 167 of the –191 to –162 rGH oligonucleotide caused a striking fourfold increase in the T_3 induction of an amputated rGH promoter. This up-mutant T_3RE was used as the basis for an evaluation of the other portions of the rGH T_3RE. Three domains were identified in this region of the rGH gene, spaced ~10 nucleotides apart, all of which were required for an optimal T_3 response. Cotransfection of excess receptor did not restore a full response of a T_3RE with a single mutation in any one of the three critical areas. On the basis of these results, a series of T_3RE "half sites" that have the sequence AGGT(C/A)A were defined in the rGH promoter. These are oriented as two direct repeats followed by an inverted repeat, the A, B, and C domains. Three such elements are present in the highly T_3-inducible rGH and α-myosin heavy-chain promoters. In the α-glycoprotein and β-TSH (thyroid-stimulating hormone) genes, only two direct repeats of this half site are found. This may have relevance as to how T_3 induces repression, as opposed to stimulation, of the transcription of these genes. Dr. Larsen's group had demonstrated earlier that T_3 does not induce the human growth hormone (hGH) promoter but that the bovine growth hormone (bGH) promoter is T_3 responsive. The latter contains sequences quite similar to the palindromic B and C domains of rGH, but the former does not.

The receptor cotransfection system was also used to demonstrate that a non-T_3–binding variant of the α_1-receptor, called α_2, cloned by Dr. William W. Chin's group (HHMI, Harvard Medical School), can interfere with the expression of T_3-dependent promoters in JEG (cotransfected with receptor) or GH4Cl cells. This protein is formed by alternate splicing of the α_1-gene transcript and binds DNA but not T_3. This interference indicated the possibility of a T_3-response–limiting function for the especially high levels of the α_2-protein in the central nervous system. It acts as a dominant negative mutation. Nuclear T_3 receptors in this unique tissue are nearly saturated in the euthyroid state, due to the type II deiodinase activity discussed earlier. The presence of the α_2-protein could serve as an alternate regulatory mechanism to regulate the T_3 response.

Dr. Larsen is also Professor of Medicine at Harvard Medical School and Senior Physician at the Brigham and Women's Hospital.

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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 (adrenaline) and norepinephrine (noradrenaline). These receptors can be categorized into two classes, termed α- and β-adrenergic receptors, which mediate the effects of catecholamines and related drugs on a wide variety of physiological processes.

I. β-Adrenergic Receptors.

A. *Molecular properties.* The laboratory continued to use techniques of site-directed mutagenesis and construction of chimeric receptors to delineate the structural basis of β-adrenergic receptor function. Studies with chimeric β_1- and β_2-adrenergic receptors indicated that multiple membrane-spanning domains contribute to the binding specificity of the β-receptors. The fourth, sixth, and seventh transmembrane helices appeared to be particularly important. In contrast, the cytoplasmic domains, especially the two ends of the third cytoplasmic loop found closest to the plasma membrane, appear to be important for determining specificity of coupling to G proteins. The most proximal region of the carboxyl-terminal tail may contribute as well.

The involvement of primarily membrane-spanning domains in contributing to the ligand-binding site was confirmed by biochemical studies in which photoaffinity and affinity-labeling reagents were covalently inserted into the ligand-binding site of either the β- or the α_2-adrenergic receptors. After appropriate digestion by chemical or enzymatic means, the peptides to which the ligands were covalently attached could be determined. These studies indicated labeling of the second transmembrane-spanning region by a covalent antagonist in the β_2-adrenergic receptor and labeling of the fourth membrane-spanning domain by both the agonist and antagonist affinity reagents for the α_2-adrenergic receptors.

The laboratory determined that a highly conserved cysteine found in the proximal portion of the carboxyl-terminal cytoplasmic tail of the β_2-adrenergic receptor is palmitoylated. This residue 343 in the human β_2-receptor is homologous with a cysteine residue in rhodopsin, which also appears to be palmitoylated. In the case of the β-receptor, mutagenesis of this cysteine leads to complete loss of the palmitoylation, as well as partial impairment of receptor coupling. Although the exact significance of this palmitoylation is unknown, palmitate in this position of the receptor may serve to attach the cytoplasmic carboxyl-terminal tail of the receptor to the plasma membrane, thus creating a fourth cytoplasmic loop.

B. *Physiological regulation of β-adrenergic receptors.* Investigation of phosphorylation of the β-adrenergic receptor by the β-adrenergic receptor kinase (βARK) during homologous desensitization has been continued. Sufficient quantities of the kinase to obtain several limited stretches of protein sequence were purified, and oligonucleotide probes were designed and used to screen a bovine brain cDNA library. Full-length clones for the βARK were obtained. The deduced sequence indicates a protein of 686 amino acids with a calculated molecular weight of just under 80,000. There is a centrally located classic protein kinase catalytic domain. This domain bears homology to all other protein kinase catalytic domains but is most similar (~33% sequence identity) to that of the cyclic nucleotide-dependent protein kinases and the C kinase family. The catalytic domain is flanked by two equal-sized domains that bear no homology to any currently sequenced protein. Messenger RNA for βARK is most abundant in brain, spleen, and heart, which are among the most heavily sympathetically innervated tissues. Southern blots reveal multiple bands hybridizing βARK probes with varying stringencies and intensities, suggesting the existence of a multigene family. Thus βARK appears to be the first sequenced member of a new gene family that may be of broad regulatory significance.

Techniques of site mutagenesis were used to create site mutants lacking either the cAMP-dependent protein kinase phosphorylation sites or the presumed sites of βARK phosphorylation at the carboxyl terminus, or both sets of sites. All of these mutants activated adenylate cyclase and bound ligands normally. However, all showed decreased agonist-promoted phosphorylation and decreased agonist-promoted desensitization under appropriate experimental conditions. Work with these mutants has provided compelling evidence that phosphorylation of the receptor by several distinct protein kinases provides the molecular mechanism

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for rapid uncoupling of the receptors during desensitization.

Dr. Lefkowitz and his colleagues described the first known inhibitors of βARK. These polyanionic compounds, such as heparin, provide relatively specific and potent inhibitors of the kinase, with the K_i of heparin ~10 nM. A permeabilized cell system was developed in which the effects of such βARK inhibitors on the process of rapid homologous desensitization could be tested. Inhibitors of βARK largely ablated this process, whereas inhibitors of the cAMP-dependent protein kinase or other protein kinases had no effect. In contrast, in this system inhibitors of protein kinase A blocked rapid heterologous desensitization.

Transcription of the gene for the β-adrenergic receptor is regulated by a variety of influences. Several classes of steroid hormones, including androgens and glucocorticoids, appear to increase the rate of transcription. The rate of transcription is also increased by cAMP. The actions of glucocorticoids and cAMP appear to be mediated by fairly typical glucocorticoid response elements and cAMP response elements, respectively, found in the 5′-untranslated region of the β_2-adrenergic receptor gene. Several of these modulators also appear to regulate the stability of the β-adrenergic receptor mRNA, providing an alternate mechanism for controlling steady-state levels of β-adrenergic receptor mRNA. Agonist stimulation *per se* also leads to decreases in β-adrenergic receptor mRNA, although the mechanism of this effect is still being worked out.

II. α-Adrenergic Receptors.

A. *α_2-Adrenergic receptors.* The two subtypes of α_2-adrenergic receptors that were cloned, the α_{2A}- and α_{2B}-receptors, were expressed in several eukaryotic expression systems. These include transient expression in COS-7 cells, as well as permanent expression in a fibroblast line. The transmembrane signaling systems to which these two distinct α_2-receptors were linked were then studied. Both subtypes potently inhibit adenylate cyclase activity and more weakly stimulate phosphatidylinositol (PI) turnover. The half-maximal effective concentration for stimulation of PI turnover is ~10-fold greater than that for inhibition of adenylate cyclase activity. Both effects are mediated by pertussis toxin-sensitive G proteins, presumably members of the G_i family. Significant differences in the biological activating properties of the two different receptor subtypes have not yet been detected.

B. *α_1-Adrenergic receptors.* The laboratory recently succeeded in cloning two new members of the α_1-adrenergic receptor subfamily of adrenergic receptors. The original α_1-adrenergic receptor that was cloned last year corresponds pharmacologically to the so-called α_{1B}-receptor. Another receptor that corresponds to the so-called α_{1A}-receptor has now been cloned. The distinction is based on pharmacological criteria. A third member of the α_1 group has recently been cloned and sequenced, and the laboratory is preparing to express this gene. Both α_{1A}- and α_{1B}-receptors appear to stimulate PI turnover potently. It remains to be seen what the biological properties of the third α_1-receptor are and what the tissue distribution of the receptors is, as determined by Northern blot analysis. Another question is whether there are additional members of the α-adrenergic receptor group that can be isolated by molecular cloning. With the advent of these new, previously unrecognized receptor subtypes comes the possibility not only for further understanding of receptor structure and function but for the development of more selective and clinically useful drugs.

Dr. Lefkowitz is also James B. Duke Professor of Medicine and Professor of Biochemistry at the Duke University Medical Center.

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TRANSCRIPTIONAL REGULATORY PROTEINS

STEVEN L. MCKNIGHT, PH.D., *Investigator*

I. CCAAT/Enhancer Binding Protein.

Two DNA sequence elements that occur quite frequently in association with promoters and enhancers are the CCAAT homology and the enhancer core homology. A heat-resistant DNA-binding activity capable of specific interaction with both of these DNA sequence elements was identified in rat liver nuclear extracts. The polypeptide that specifies this activity, termed CCAAT/enhancer binding protein (C/EBP), was purified in the McKnight laboratory by Drs. Barbara Graves, Peter Johnson, and William Landschulz, allowing derivation of a partial amino acid sequence. The amino acid sequence was used to generate specific antibodies, which were in turn used to retrieve a molecular clone of the gene that encodes C/EBP.

A. *Tissue distribution.* The tissues and cell types that express C/EBP have been identified by a combination of antibody staining and nucleic acid hybridization assays. High levels of C/EBP and mRNA were observed in adipose, liver, placenta, lung, adrenal gland, and intestine. Each of these tissues metabolizes lipids at an exceptionally high rate, leading to the hypothesis that C/EBP might be a general regulator of proteins and enzymes involved in lipid synthesis. Evidence favoring this interpretation has begun to emerge from several different experimental approaches that have been conducted in Dr. McKnight's laboratory and the laboratory of Dr. M. Daniel Lane (The Johns Hopkins University Medical School). Purified C/EBP binds to the promoters and enhancers of a number of genes that are expressed selectively in adipose and liver. These include the genes encoding serum albumin (the major lipid carrier protein), stearoyl acyl-CoA desaturase (SCD1), and 422/aP2 protein (an intracellular lipid carrier protein). The significance of such binding is strongly substantiated by transient transfection assays, in which a C/EBP expression vector has been found to be capable of trans-activating each of the aforementioned genes.

B. *Structural properties of the C/EBP DNA-binding domain.* The amino acid sequence of C/EBP is similar to the sequences of several transforming proteins, including Fos, Jun, and Myc. This region of sequence relatedness, which is located within the DNA-binding domain of the C/EBP polypeptide, is free of amino acid residues that are incompatible with α-helical structure. It also contains a heptad repeat of leucine residues. On the basis of these properties, Drs. Landschulz, Johnson, and McKnight speculated that this region of C/EBP might form an amphipathic α-helix and that the hydrophobic surface of the helix would be used as a dimerization interface to bring two polypeptide chains together. This leucine zipper motif is characteristic of a newly discovered class of sequence-specific DNA-binding proteins. Evidence supportive of the leucine zipper model has emerged from mutational and spectroscopic studies.

II. Herpes Simplex Virus Protein 16.

The lytic infectious cycle of herpes simplex virus (HSV) is characterized by a three-tiered cascade of viral gene expression. During the first 3 h postinfection, five viral genes are transcribed. These five immediate early (IE) genes encode regulatory proteins that play a critical role in the expression of the subsequent class of viral genes, the delayed early (DE) genes. DE genes, which are expressed between 3 and 9 h postinfection, encode enzymes and proteins necessary for the replication of viral DNA. The expression of the final class of viral genes, the late (L) genes, requires DNA replication and occurs between 9 and 18 h postinfection. Most of the L genes encode structural proteins that form the mature virion. However, the product of one L gene, HSV protein 16 (VP16), plays a regulatory role. When a virion enters a newly infected cell, VP16 moves to the nucleus and serves as a potent activator of IE gene expression.

A. *VP16 attaches to the enhancers of IE genes indirectly.* The DNA sequence elements of IE genes that are required for trans-activation by VP16 have been mapped and shown to represent binding sites for cellular DNA-binding proteins. Thus VP16 appears to "piggyback" its way onto IE genes via cellular transcription factors. One of the cellular proteins critical for VP16 function, octamer transcription factor 1 (OTF1), has been studied extensively by Dr. Winship Herr and his colleagues at Cold Spring Harbor Laboratory. A second protein, immediate early facilitator GA (IEFga), has been purified by Dr. Karen LaMarco in the McKnight laboratory. Once attached to an IE enhancer via these cellular pro-

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teins, VP16 triggers activation of gene expression by use of a highly acidic segment of 60 amino acids located at its carboxyl terminus. The mechanism by which this acidic domain activates transcription is unknown.

B. *Derivation of a trans-dominant form of VP16.* During the course of molecular genetic characterization of VP16, Dr. Steven Triezenberg and Dr. McKnight observed that truncated forms of the protein lacking the acidic activation domain impede the function of normal VP16. Dr. Alan Friedman and Dr. McKnight extended these observations by preparing a cell line that constitutively expresses an activation-defective form of VP16. The truncated VP16 is located in the nucleus of these cells. These cells exhibit substantial immunity when challenged by infecting HSV. The cause of the observed immunity has been traced to an impediment in the activation of IE genes. Drs. Friedman and McKnight, in collaboration with Dr. Edward Birkenmeier of the Jackson Laboratory, recently succeeded in generating a transgenic mouse that expresses the truncated form of VP16. Experiments are under way to test whether such procedures have rendered the transgenic mice immune to HSV infection.

Dr. McKnight is also a staff member of the Department of Embryology at The Carnegie Institution of Washington and Adjunct Professor in the Departments of Biology and of Molecular Genetics at The Johns Hopkins University.

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REGULATION OF THE ADRENAL STEROIDOGENIC ENZYMES

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

Dr. Parker's laboratory is studying the mechanisms that control the gene expression of the adrenal steroidogenic enzymes. Steroid biosynthesis, which involves the conversion of cholesterol to biologically active products, requires the concerted action of five cytochrome P-450 enzymes. Certain of these enzymes, such as the side-chain cleavage enzyme (SCC), are expressed in all steroidogenic tissues; others, notably steroid 21-hydroxylase (21-OHase) and 11β-hydroxylase (11β-OHase), are expressed only in the adrenal cortex. Treatment of adrenocortical cells with the trophic hormone ACTH coordinately increases the expression of all of these genes via the cAMP second messenger system. This induction represents a major component of the hormone's action to maintain adrenocortical competence for steroid biosynthesis. The objective of these studies is to define the factors that regulate the expression of the adrenal steroidogenic enzymes. A central hypothesis is that shared factors determine the ACTH induction and adrenal-specific expression of this network of genes, whereas unique elements permit SCC expression in other steroidogenic tissues.

I. 21-Hydroxylase Gene Regulation.

Initial studies focused on 21-OHase. Transfection experiments showed that all elements essential for tissue-specific and cAMP-inducible expression were found within 330 bp of the transcription start site. Functional and DNase I footprinting experiments defined a complex array of regulatory elements within these 330 bp. Two of these elements, at −65 and −215, share the heptamer AGGTCAG and bind the same, or highly related, protein(s). Moreover, both elements increased the activity of a heterologous promoter in an adrenal-specific manner.

Ongoing studies are directed at characterizing the proteins that interact with these elements to regulate 21-OHase transcription. These studies, which will focus on the protein that interacts with the AGGTCAG motif, will utilize two complementary approaches: direct purification of the regulatory proteins and screening of expression libraries with oligonucleotide probes. To provide sufficient material to make the first approach possible, methods have been developed for making whole-cell extracts from bovine adrenal glands. These preparations appear to contain most, if not all, of the proteins previously characterized in Y1 cells. The first step in the expression screening of cDNA libraries with oligonucleotide probes is the construction of the libraries in λgt11. Such libraries have been prepared from both mouse adrenal RNA and Y1 cell RNA, and screening with oligonucleotides is now in progress.

To extend their investigations of 21-OHase expression in Y1 cells, Dr. Parker and his colleagues recently initiated a collaboration with Drs. Jonathan G. Seidman (HHMI, Harvard Medical School) and David Milstone to study expression of 21-OHase in transgenic mice. These experiments suggest that additional regulatory elements that are not required for expression in Y1 cells are required for 21-OHase expression in transgenic mice. After the region that controls this effect is mapped, studies will be performed to determine if any adrenal-specific differences, such as DNase I–hypersensitive sites or differences in methylation status, are unique to that region.

II. 11β-Hydroxylase Gene Regulation.

To complement and extend the studies with 21-OHase, the laboratory cloned and characterized the 11β-OHase gene. As was the case for 21-OHase, the 5′-flanking region of 11β-OHase directed adrenal-specific and cAMP-induced expression of a linked reporter gene. Potential regulatory elements within the 11β-OHase promoter region were defined by DNase I footprinting experiments with nuclear extracts from Y1 adrenocortical cells. One interaction, centered at −52, involved a sequence that closely resembled the consensus sequence of the cAMP-responsive element, an element found in the promoter region of many cAMP-responsive genes. Extensive studies showed that this element, which is essential for 11β-OHase expression, determines cAMP responsiveness of the gene in a manner analogous to the cAMP-responsive element defined in other systems. DNase I footprinting analyses defined two other elements, one centered at −310 and one at −370. Mutation of either sequence significantly inhibited promoter activity. Moreover, oligonucleotides containing either element restored activity, in an orientation-independent manner, to a promoter fragment containing 11β-OHase sequences from −40. Examination of the sequence of the 5′-flanking region of the 11β-OHase gene

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also revealed sequences at −249 and −148 that resemble the sequence motifs at −210 and −65 in the 21-OHase promoter region. However, mutagenesis of these elements showed that they do not play significant roles in 11β-OHase expression. These studies have thus identified three regulatory elements that play important roles in 11β-OHase expression. Efforts are now under way to isolate and characterize the proteins that interact with the two upstream elements.

III. Side-Chain Cleavage Enzyme Gene Regulation.

In a new direction, the laboratory recently cloned the mouse SCC gene and began to study its regulation. This gene is expressed by all steroidogenic tissues in a cAMP-responsive fashion. The promoter region of the SCC gene directed high levels of expression of a linked reporter gene after transfection into Y1 adrenal cells; this expression was increased fourfold by treatment with 8-Br-cAMP. Several regions that are important for expression were localized by 5′-deletion experiments, including the regions from −420 to −318 and from −200 to −75. DNase I footprinting experiments revealed several protein-DNA interactions. The sequence protected in one footprint, at −70, shares the AGGTCA motif found in the 21-OHase elements at −210 and −65. A second element, at −42, is an inverted repeat of the central region of a 21-OHase regulatory element at −140. Roles of these elements in SCC expression have not yet been demonstrated, and functional studies are in progress to address this issue. However, the importance of these motifs in 21-OHase expression predicts that they may also play major roles in SCC expression. This would provide the first evidence for the model that shared regulatory elements are involved in coordinate regulation of the steroidogenic enzymes.

Collectively these studies have defined important regulatory elements that control the expression of the adrenal steroidogenic enzymes. Support for the model that shared elements mediate the coordinate expression of these genes is provided by the identification of two elements that are present in the promoter regions of both 21-OHase and SCC. The successful isolation of the regulatory proteins that interact with these elements and the analysis of their regulation will provide insights into the mechanisms that determine the complex regulation of this essential group of genes.

Dr. Parker is also Assistant Professor of Medicine and of Biochemistry 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 of the laboratory 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.

I. Phosphoinositide Metabolism.

A. *Phosphatidylinositol kinase.* Previous work has shown that EGF stimulates the activity of a PI kinase in A431 cells, a human epidermal carcinoma cell line. This activation is retained in membranes prepared from EGF-treated cells. The EGF-stimulated PI kinase was purified from A431 cells and shown to be a 55 kDa monomeric protein. Because cultured cells provide only limited amounts of material for purification, the PI kinase has recently been purified from human placenta. With this material, peptides were produced by cleavage with cyanogen bromide, and the sequence of an extremely hydrophobic peptide was determined.

Polyclonal antibodies were raised to the purified PI kinase. The antibodies identify a 55 kDa protein on Western blots. These antibodies were used to screen a λgt11 human placental cDNA library. Twenty-eight putative positive cDNA clones were selected by this procedure. The protein sequence obtained from the placental enzyme was used to synthesize oligonucleotide probes and to rescreen the antibody-positive clones and the library. All positive clones will be further characterized. Sequence information obtained from this cloning work will provide useful structural information on the PI kinase. An analysis of the PI kinase sequence will provide information on the relationship of this enzyme to other known families of small-molecule kinases or protein kinases.

The PI kinase is stimulated in cells treated with EGF. In addition, the activity of the enzyme is enhanced in cells treated with the phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate (TPA), a known activator of protein kinase C. Treatment of A431 cell membranes with protein kinase C also leads to the activation of PI kinase activity, which supports a role for protein kinase C in the regulation of PI kinase activity. Despite the fact that both EGF and protein kinase C appear to activate the PI kinase, incubation of the purified PI kinase with either protein kinase C or the EGF receptor kinase under phosphorylating conditions leads to neither phosphorylation nor activation of the PI kinase. These data suggest that a third component is required to mediate the effects of EGF and protein kinase C on the PI kinase. Work is in progress to reconstitute activation of the PI kinase *in vitro* by adding back various extracts from A431 cells.

B. *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 have not been characterized. A PIP phosphatase from A431 cells has been partially purified and characterized in Dr. Pike's laboratory. It appears to be specific for dephosphorylating PIP, as other phosphoinositides or inositol phosphates that have phosphate groups in position 4 of the inositol ring do not appear to function as alternate substrates or competitive inhibitors. Although protein phosphatases do not dephosphorylate PIP, the PIP phosphatase is inhibited by some common protein phosphatase inhibitors, such as fluoride, zinc ions, and sodium orthovanadate. Physical and biochemical studies suggest that PIP phosphatase is a transmembrane glycoprotein with a molecular weight of ~140,000. Future work will concentrate on the purification of the enzyme and the generation of antibodies and protein sequence necessary for cloning of PIP phosphatase.

II. Desensitization of the EGF Receptor.

Previous work in the laboratory has shown that treatment of A431 cells with high concentrations of EGF reduces the ability of the treated cells to internalize ^{125}I-EGF. This desensitization of ligand-in-

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duced receptor internalization occurred in cells in which protein kinase C activity had been downregulated by prolonged treatment with phorbol esters, implying that the process is independent of protein kinase C. Pretreatment of the cells with compounds such as trifluoperazine, chlorpromazine, and W7 blocks the effects of EGF on hormone internalization.

Additional studies suggest that several other EGF-stimulated responses are also desensitized after treatment of A431 cells with high concentrations of EGF. These include EGF-stimulated PI turnover and EGF-stimulated tyrosine protein kinase activity. Desensitization of EGF-stimulated PI turnover occurred rapidly at 37°C, with half-maximal effect at 15 min. The desensitization was homologous, or agonist specific, in nature. Treatment of cells with EGF decreased the responsiveness of the cells to EGF, whereas treatment with bradykinin (another compound capable of stimulating PI turnover in these cells) did not alter the ability of the cells to respond to EGF. Like desensitization of ^{125}I-EGF internalization, desensitization of EGF-stimulated PI turnover occurred in cells that had been rendered protein kinase C–deficient by prolonged incubation with TPA. This again suggests that desensitization of the EGF receptor is not mediated by protein kinase C. Treatment of the cells with trifluoperazine could block desensitization of PI turnover.

The EGF receptor is present on the cell surface as a monomer. Upon binding EGF, the monomeric receptor dimerizes; it is this dimeric form of the receptor that has been hypothesized to be involved in signal transduction. Studies in Dr. Pike's laboratory have shown that EGF receptor dimerization is inhibited in desensitized cells. This is true both when whole cells are treated with EGF and the monomers and dimers isolated by sucrose density gradient centrifugation and when the capacity of isolated monomers to form dimers is assessed *in vitro*.

Not all treatments that affect EGF receptor sensitivity lead to alterations in receptor dimer formation. Treatment of cells with TPA stimulates the activity of protein kinase C, which phosphorylates the EGF receptor. This leads to a loss of EGF binding and a decrease in the ability of EGF to stimulate responses via its receptor. However, treatment of cells with TPA does not alter the ability of EGF to induce EGF receptor dimers, indicating that the effects on dimerization are specific for EGF-dependent desensitization.

In many cases desensitization of receptors has been associated with the phosphorylation of the receptor by cytosolic kinases. Dr. Pike's laboratory has therefore begun to investigate the possibility that a cytosolic kinase in A431 cells is capable of phosphorylating and desensitizing the EGF receptor. Preliminary data show that A431 cells contain a cytosolic kinase that is capable of phosphorylating affinity-purified EGF receptors. The activity of the enzyme appears to be stimulated by EGF and inhibited by trifluoperazine. Studies are in progress to purify and characterize this kinase and determine whether it is involved in desensitization of the EGF receptor.

Dr. Pike is also Assistant Professor of Biochemistry and Molecular Biophysics at the Washington University School of Medicine.

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Article

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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 thrombosis and how this balance is disrupted in many human diseases. Dr. Sadler has concentrated on two areas: 1) von Willebrand factor and von Willebrand disease and 2) the regulation of blood clotting reactions by endothelial cells and monocytes.

I. Molecular Biology of Human von Willebrand Factor and von Willebrand Disease.

Von Willebrand factor is a multimeric plasma glycoprotein that is required for platelet adhesion to sites of injury and for normal survival of factor VIII in the circulation. Deficiency of von Willebrand factor (von Willebrand disease) may cause bleeding that resembles platelet dysfunction or hemophilia. Von Willebrand disease is the most common inherited bleeding disorder of humans and is phenotypically heterogeneous. Knowledge of the molecular defect in variants of von Willebrand disease will illuminate structure-function relationships of von Willebrand factor and will assist the development of improved therapy.

A. *Structure of the von Willebrand factor gene.* The von Willebrand factor gene was cloned, and its structure was determined by restriction mapping and DNA sequencing. The gene spans ~178 kb and contains 52 exons. The von Willebrand factor subunit has a highly repeated structure, and the gene segments that encode homologous domains are similar in structure. This is consistent with the evolution of the protein by gene duplication or exon shuffling. Some features of the gene structure have found immediate clinical use. A polymorphic BKm (banded krait minor) repeat or VNTR (variable number of tandem repeats) region was identified in intron 40 and was used as a marker to diagnose severe von Willebrand disease in a fetus for whom the known RFLP (restriction fragment length polymorphism) markers were not informative.

Knowledge of the gene structure permitted Dr. Sadler to determine the molecular defect in several patients with severe von Willebrand disease type III. Deletions of the entire gene were found in two unrelated patients. Three other patients were shown to have partial gene deletions. One of these patients had a small homozygous deletion that included only a single exon. These patients are unusual because they have produced alloantibody inhibitors of transfused von Willebrand factor. In 27 families with von Willebrand disease type III that do not have alloantibody inhibitors, no gene deletions have been found. In contrast, in 10 families with alloantibody inhibitors, six deletions have been identified. Thus gene deletions may predispose patients to this rare complication of therapy.

B. *Structure of the von Willebrand factor pseudogene.* In addition to the von Willebrand factor gene on chromosome 12, a partial unprocessed pseudogene on chromosome 22 was cloned and sequenced. The pseudogene corresponds to exons 23–34 of the authentic gene. The von Willebrand factor gene and pseudogene differ in sequence by only ~3%, which suggests a recent origin for the pseudogene. With this structural information, gene sequences can now be amplified selectively and characterized without interference from the pseudogene.

II. Regulation of Blood Coagulation by Endothelial Cells and Monocytes.

The endothelium and monocytes normally do not promote blood clotting reactions. However, inflammatory mediators such as interleukin-1 and tumor necrosis factor-α (TNF-α) can induce these cells to stimulate blood clotting, thereby linking the initiation of blood coagulation to activation of the immune system. Dr. Sadler has studied three hemostatic proteins that are regulated in endothelial cells by inflammatory mediators: plasminogen activator inhibitor-2, tissue factor, and thrombomodulin.

A. *Plasminogen activator inhibitor-2 (PAI-2).* Blood clot destruction may be initiated by urokinase or tissue-plasminogen activator. In turn these proteases are regulated by several plasminogen activator inhibitors. PAI-1 is characteristic of endothelial cells; PAI-2 is found mainly in placenta and monocytes. PAI-2 is induced in monocytes by inflammatory mediators and may regulate fibrin deposition that accompanies cell-mediated immune responses.

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PAI-2 was cloned in Dr. Sadler's laboratory and shown to be a new member of the serine protease inhibitor gene family that is particularly similar to chicken ovalbumin. The human PAI-2 gene has now been cloned and its structure determined. Six of the seven intron positions in the PAI-2 gene are identical to those in the ovalbumin gene. Thus PAI-2 is the closest known mammalian homologue of avian ovalbumin.

Ovalbumin is unusual among eukaryotic proteins because it is secreted efficiently without cleavage of an amino-terminal signal peptide. The similarity of PAI-2 to ovalbumin suggested that PAI-2 might share this property. This was confirmed by study of PAI-2 synthesis in a human monocytic cell line. It is not known whether this has significance for the physiological function of PAI-2 or is only a fortuitous consequence of the homology between PAI-2 and ovalbumin.

Endothelial cells were known to make PAI-1 but were generally thought not to express PAI-2. Dr. Sadler has shown that the levels of PAI-2 mRNA and protein are increased in endothelial cells by either phorbol esters or TNF-α. This occurs without a detectable change in PAI-2 transcription. The mRNA for PAI-2 is unstable in endothelial cells but is stabilized by protein synthesis inhibitors and appears to be stabilized transiently by treatment of cells with phorbol esters or TNF-α. Thus PAI-2 may be induced in part by inhibition of mRNA degradation.

B. *Tissue factor.* Tissue factor is a membrane receptor that binds coagulation factor VII, and the tissue factor–factor VII complex is the most important physiological activator of blood coagulation. Tissue factor is found in many tissues but is not normally expressed by cells that are in contact with blood. However, exposure of endothelium or monocytes to inflammatory mediators such as TNF-α rapidly induces tissue factor, and this apparently contributes to the pathophysiology of disseminated intravascular coagulation.

Human tissue factor was cloned in Dr. Sadler's laboratory, the gene was localized to human chromosome 1, and RFLPs with *Msp*I and *Taq*I were identified. These cDNA probes were used to study the regulation of tissue factor in cultured endothelial cells. As was found for PAI-2, tissue factor mRNA is unstable but can be stabilized by inhibitors of protein synthesis. Unlike PAI-2, tissue factor gene transcription is induced by phorbol esters or TNF-α. Thus tissue factor is regulated at the level of transcription and also potentially at the level of mRNA stability. The structural elements required for this regulation are under study.

C. *Thrombomodulin.* Thrombomodulin is an endothelial cell membrane receptor that binds thrombin and alters its substrate specificity. This activity serves an essential anticoagulant function. Thrombomodulin is expressed in endothelial cells of most blood and lymphatic vessels. Thrombomodulin expression is decreased by inflammatory mediators such as TNF-α and interleukin-1, and this may contribute to intravascular thrombosis at sites of inflammation.

Human thrombomodulin cDNA clones were isolated in Dr. Sadler's laboratory, and the gene was localized to chromosome 20cen-p12. These clones have been employed to study the regulation of thrombomodulin in cultured endothelial cells. The thrombomodulin gene appears to be transcribed constitutively and to be unaffected by phorbol esters or TNF-α. Treatment of cells with phorbol esters does not alter thrombomodulin activity or mRNA levels. However, treatment with TNF-α for 24 hours causes an ~90% reduction in total and cell surface thrombomodulin, with no apparent decrease in mRNA. These data suggest that thrombomodulin is not regulated significantly by controlling mRNA concentration but must be regulated during or subsequent to translation.

Thrombomodulin presumably interacts with membrane or cytoskeletal components to undergo endocytosis and degradation in response to TNF-α. Recombinant thrombomodulin was expressed in COS-7, CV-1, and K562 cells and appeared to be identical structurally and functionally to nonrecombinant thrombomodulin. Whether endocytosis requires specific sequences in the cytoplasmic tail or in other domains of thrombomodulin is under investigation by the expression of thrombomodulin mutants in such heterologous cells.

Further study of tissue factor, PAI-2, and thrombomodulin will clarify the endothelial cell's role in inflammation and hemostasis and may suggest a strategy for altering the expression of individual proteins to achieve a therapeutic effect in patients with bleeding or thrombosis.

Dr. Sadler is also Associate Professor of Medicine and Assistant Professor of Biochemistry and Molecular Biophysics at the Washington University School of Medicine.

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PATHOGENESIS OF INFECTIOUS AGENTS

GARY K. SCHOOLNIK, M.D., *Associate Investigator*

Two areas of investigation are being pursued in Dr. Schoolnik's laboratory: 1) the pathobiology of infections of mucous membranes and 2) the immunochemistry of small peptides.

I. Pathobiology of Mucosal Infections.

Previous studies have focused on the molecular mechanisms by which microorganisms bind host epithelial cells that line the surfaces of the respiratory, gastrointestinal, and genitourinary tracts. This process is mediated in most instances by a proteinaceous appendage of the microorganism, termed *adhesin*, and the corresponding epithelial cell receptor to which the adhesin binds in a stereoscopically specific manner. In the past year two related aspects of this binding event have been studied. First, surface-exposed adhesin proteins exhibit antigenic diversity, presumably to evade the host immune response; the genetic and structural basis for this phenomenon has been investigated. Second, after the adhesin-mediated attachment of the microbe to the epithelial cell, penetration of the bound cell by invasive microorganisms ensues; this process is mediated in some bacteria by a second microbial protein, termed *invasin*. The structure and function of one such invasin was studied.

A. *Antigenic diversity of microbial adhesin proteins.* Several bacterial species that are pathogenic but taxonomically diverse, including *Neisseria gonorrhoeae*, *Pseudomonas aeruginosa*, *Bacteroides nodosus*, *Vibrio cholerae*, and *Moraxella bovis*, produce specialized adhesin proteins or pili, which radiate from the bacterial surface as thin, flexible filaments; each filament is composed of multiple, identical repeating subunits of ~20 kDa. The amino acid sequences of the pili subunits of the above-noted bacterial species are related but not identical: the amino terminus of each is an unusual amino acid, *N*-methylphenylalanine, and their sequences are nearly homologous for the first 45 residues. Thereafter their primary structures diverge. Genetic mechanisms for the production of antigenic diversity and the extent of the diversity have been evaluated for several of these species. The genome of *P. aeruginosa*, a species that exists most often as a free-living organism in water, contains a single pili subunit gene; antigenic diversity arises as a result of accumulated point mutations. Thus this species, which infrequently contends with the host immune system, has a limited capacity for the expression of antigenic diversity. In contrast, *N. gonorrhoeae* exists only on human mucous membranes, is continually exposed to the selective pressures of the immune system, and possesses a genetic mechanism for the production of a large number of pili antigenic variants. The basis for this diversity is the presence of multiple variant pili subunit gene sequences in the genome of each strain, each variant sequence coding for hypervariable regions of the subunit that specify unique, immunodominant antigenic determinants. Expression of an antigenic variant occurs when the corresponding pili subunit gene sequence is recombined into an expression locus yielding a gene conversion event that links sequences coding for the conserved amino-terminal domain with sequences coding for the hypervariable carboxyl-terminal domain of the pili subunit.

Intermediate with respect to pili antigenic diversity between *P. aeruginosa* and *N. gonorrhoeae* is *M. bovis*, the etiologic agent of bovine keratoconjunctivitis. *M. bovis* exists in nature in three ecological niches: as a pathogen of bovine conjunctival membranes, where it is exposed to IgA and IgG in tears; as a commensal bacterium of the face fly; and as a slowly replicating organism in fomites such as dust. Thus in one ecological niche (ocular membranes) the expression of pili antigenic variants by *M. bovis* may be essential for its survival, whereas in another (e.g., dust) it may be irrelevant. In contrast to *N. gonorrhoeae*, in which a single strain can sequentially express many pili antigen variants, and *P. aeruginosa*, in which a single strain expresses only one pili antigenic type, the expression of pili by *M. bovis* oscillates between two pili antigenic variants, α and β; simultaneous expression of both pili types does not occur.

The complete α and β *M. bovis* pili amino acid sequences were determined, compared, and found to be ~70% homologous, indicating that they are the products of two separate but related genes. Regions of sequence heterogeneity were found to specify α- or β-specific pili epitopes, since the two proteins exhibit only 50% shared antigenicity. Pathogenicity studies in calves of α- or β-pili expressing variants of the same strain indicated that β-pili confer or are associated with a relative advantage during the first stages of ocular infection. The

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genetic mechanism for the oscillation of α- versus β-pili expression was sought; the α- and β-pili subunit genes were found to arise from a single genetic locus by a process of chromosomal rearrangement that occurs as an inversion event in which the end parts of the inverted segment are within the pili subunit structural genes. Thus *M. bovis* possesses a genetic mechanism for pili expression that restricts the antigenic repertoire of each strain to two types only. When these findings are considered in evolutionary terms, it seems likely that a primordial pili gene of the *N*-methylphenylalanine class entered several different bacterial species; once established in each species, distinctive mechanisms for the production of antigenic diversity arose as a consequence of the ecology of the organism and the selective pressures of the host immune response.

B. *Characterization of an invasin protein of* Yersinia enterocolitica. Invasion of epithelial cells is an important step in the pathogenic strategy of many bacterial species. In two of the three species comprising the genus *Yersinia* (*Y. pseudotuberculosis* and *Y. enterocolitica*) the invasive phenotype is confined by a single outer membrane protein, invasin. In studies carried out collaboratively with the laboratory of Dr. Stanley Falkow (Stanford University), the gene coding for the invasin of *Y. enterocolitica* was cloned, sequenced, and the topography of the protein on the surface of the bacterium determined. The gene for invasin codes for an 835–amino acid protein; the use of antibodies to synthetic peptides corresponding to various regions of this sequence to search for surface-exposed domains of invasin indicated that the amino-terminal and carboxyl-terminal domains of the protein are embedded within the outer membrane, leaving a centrally located, surface-exposed loop containing the receptor binding domain; within this loop there exists a serine protease-sensitive region that is normally cleaved during the invasive process.

Studies to define the cellular receptor for invasin, to determine the minimal sequence constituting the receptor binding domain, and to learn how invasin initiates phagocytosis by all eukaryotic cells (except erythrocytes) are being conducted.

II. Immunochemistry of Small Peptides.

Small, synthetic peptides containing amino acid sequences found in microbial proteins have been proposed as vaccines for the prevention of a remarkable variety of viral, bacterial, and parasitic infectious diseases. However, in practice most such vaccines lack efficacy; although they elicit antisera that bind the immunizing peptide with high affinity, the affinity of the same sera for the target protein is often substantially lower, apparently below the affinity required to confer a protective effect. An immunochemical explanation for this phenomenon was sought, using as a model system antisera elicited to a peptide corresponding to a 13–amino acid segment of the central α-helix of bovine calmodulin. The results of these studies indicate that antibodies were elicited to at least two distinct regions of the peptide. Antibodies of one specificity within the polyclonal immune response to the peptide do not require the carboxyl-terminal two amino acids of the peptide for antigenic recognition, are present in the sera in relatively low concentrations, and are able to bind the target protein (calmodulin) with high affinity. Antibodies of the other antibody specificity require atomic information contributed by the carboxyl-terminal two residues of the peptide, are present in the sera in relatively high concentrations, but bind the target protein with low affinity. The implications of this study for the development of peptide vaccines stem from the recognition that within a polyclonal, anti-peptide immune response may exist a small population of antibodies that cross-react with the target protein with high affinity. Studies are being conducted on the construction of peptide immunogens designed to maximize the proportion of useful antibodies within a polyclonal immune response.

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

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ADENOVIRUS TRANSCRIPTIONAL CONTROL

THOMAS SHENK, PH.D., Investigator

I. Transcriptional Activation by Adenovirus E1A Gene Products.

The E1A gene is the first adenovirus gene to be expressed when the viral chromosome reaches the nucleus of the infected cell. E1A gene products then activate transcription of all the remaining early viral genes and further activate expression of the E1A gene itself. The mechanism by which E1A products activate gene expression remains unclear. However, several cellular transcription factors that play roles in the process have now been identified.

Insight to the factors involved in transcriptional activation came initially from the identification of specific factor binding sites within the control regions of early viral genes. One such binding site is the cAMP response element (CRE). This is the recognition site for transcription factors that have been termed CRE-binding protein (CREB) and activating transcription factor (ATF). CREB/ATF activities probably represent a family of DNA-binding proteins that recognize the same DNA sequence. Binding sites for these activities were shown to be present in the adenovirus E1A, E2, E3, and E4 transcriptional control regions.

The presence of CREB/ATF-binding sites predicted that early viral transcription should be responsive to cAMP. This proved to be the case. E1A gene products and cAMP acted in synergy to induce transcription in virus-infected cells. For example, if the level of E4 gene transcription in the absence of either cAMP or E1A gene products is set to 1, addition of either cAMP or E1A products alone induced transcription by factors of ~5 or 10, respectively, while addition of both induced transcription by a factor of ~200.

Transfection experiments demonstrated that the transcriptional response to cAMP plus E1A products required a functional CREB/ATF-binding site. Point mutations within the binding site markedly reduced the response to the activators. Curiously, however, cAMP plus E1A products did not induce any detectable quantitative or qualitative change in CREB/ATF-binding activity. A different transcription factor, AP-1, was altered. The level of AP-1 activity was modestly induced by cAMP alone, and it increased to significantly higher levels upon treatment with cAMP in the presence of E1A products. The consensus AP-1–binding site [5′-TGA(C/G)TCA-3′] differs from the recognition sequence for CREB/ATF (5′-TGACGTCA-3′) by only 1 base pair. The induced AP-1 activity bound efficiently to a variety of both AP-1– and CREB/ATF-binding sites present in E1A-inducible promoters. The maximal induction of AP-1 DNA-binding activity in infected cells required E1A proteins, cAMP, functional protein kinase A, and active transcription. The cytoplasmic levels of both c-fos and junB mRNAs were rapidly increased by cAMP treatment and increased to substantially higher levels by cAMP treatment in the presence of E1A proteins. It is likely that the AP-1 activity under study comprises c-fos and junB polypeptides.

These results suggest that E1A products in the presence of cAMP induce transcription of early viral genes in part by causing an increase in the level of AP-1 activity within infected cells. The mechanism underlying the increase in AP-1 activity is presently under study.

II. Modification of a Transcription Factor in Response to an Adenovirus E4 Gene Product.

E2F is a cellular, sequence-specific DNA-binding factor that was originally identified in adenovirus-infected cells and shown to interact with pairs of sites that occur upstream of the adenovirus E1A and E2 early mRNA cap sites. The laboratory has demonstrated that substantial quantities of E2F activity are present in uninfected as well as infected cells. The binding properties of E2F were altered by adenovirus infection. E2F from infected cells bound cooperatively to the pair of sites within the E2 control region, while the factor from uninfected cells bound to each of the two sites independently. Cooperative binding was sensitive to the spacing between the sites and their relative orientation.

Production of the infection-specific E2F activity was dependent on a product of the adenovirus E4 gene. Analysis of E2F activity in cells infected with mutant viruses revealed that the E4 6/7 ORF, which encodes a 17 kDa polypeptide, was essential for modification of E2F activity to permit cooperative binding.

The infection-induced change in E2F activity proved to be important for maximal E2 early mRNA accumulation in some cell lines but not others. Apparently the E4-induced alteration in E2F activity provides an alternative activation pathway, in addition to that mediated by E1A products, for the E2 early gene. That is, both E1A and E4 gene products

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can independently contribute to activation of E2 transcription.

The nature of the modification that alters E2F-binding properties and the effect of cooperative binding on E2F function are presently under study.

Dr. Shenk is also James A. Elkins, Jr., Professor of 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) supports the growth, differentiation, and survival of mononuclear phagocytes by binding to a high-affinity cell surface receptor (CSF-1R) encoded by the c-*fms* proto-oncogene. CSF-1R is expressed on monocytes, macrophages, and their committed precursors, as well as on placental trophoblasts during fetal development. Ligand binding activates the receptor tyrosine-specific protein kinase, leading to phosphorylation of cellular substrates that convey mitogenic signals from the plasma membrane to the cell nucleus. The major goals of this laboratory have been to understand the mechanism of receptor kinase activation, to identify substrates of the kinase that play a physiologic role in signal transduction, and to pinpoint genetic alterations in the c-*fms* gene that constitutively activate the CSF-1R kinase and induce oncogenic transformation.

I. Transforming Potential of Human Colony-stimulating Factor-1 Receptor.

Human CSF-1R is an integral transmembrane glycoprotein consisting of a 512–amino acid extracellular ligand-binding domain, a 25–amino acid transmembrane segment, and a 435–amino acid cytoplasmic tyrosine kinase domain. Recombination of cat c-*fms* sequences into feline sarcoma virus (FeSV) is responsible for its oncogenicity. Although analogous in structure to CSF-1R, the retroviral v-*fms* oncogene encodes a variant glycoprotein that exhibits ligand-independent tyrosine kinase activity.

This year Dr. Sherr's laboratory identified critical genetic alterations in the human c-*fms* proto-oncogene that unmask its oncogenic potential. Homologous recombination in lambda phage was used to generate chimeric receptor genes between feline v-*fms* and human c-*fms* cDNAs. When expressed in mouse NIH 3T3 cells, glycoproteins containing residues 1–308 of human CSF-1R fused to the remainder of the v-*fms*–coded glycoprotein functioned as normal receptors, whereas reciprocal chimeras elicited ligand-independent cell transformation. Thus "activating mutation(s)" were predicted to reside within the first 308 v-*fms* codons. Based on differences in the nucleotide sequences of the feline v-*fms* and c-*fms* genes in this region, human c-*fms* mutants were generated and tested for transforming activity. Foci of transformed cells were induced by receptors bearing a serine-for-leucine substitution at codon 301. Although located in the CSF-1R extracellular domain, the activating mutation did not affect the high-affinity ligand-binding site, so that the growth of transformed cells was further stimulated by CSF-1. Truncations or mutations affecting the distal CSF-1R carboxyl-terminal tail, although themselves insufficient to activate oncogenicity, enhanced the transforming efficiency of receptors containing the serine 301 mutation. Therefore multiple genetic events within c-*fms* can collaborate to generate a fully transformed phenotype.

Analogous mutations within the c-*fms* proto-oncogene *in situ* might contribute to leukemias involving cells of the mononuclear phagocyte series. Monoclonal antibodies to extracellular epitopes in human CSF-1R, developed in Dr. Sherr's laboratory, were used to detect receptor expression on leukemic blasts from ~40% of pediatric and adult acute myelogenous leukemia (AML) patients. Surprisingly, this did not correlate with other characteristics of monocyte differentiation. The possibility that c-*fms* genes in receptor-positive cases have acquired activating mutations is under investigation. Four monoclonal antibodies inhibited CSF-1 binding to its receptor, and one inhibited the ligand-independent growth of NIH 3T3 cells transformed by activated c-*fms* genes without affecting CSF-1R internalization or degradation. The latter result suggests that mutations that activate the CSF-1R kinase alter the aggregation state of the receptor at the cell surface.

II. Genomic Organization of the Human c-*fms* Gene.

The complete nucleotide sequence of the human c-*fms* gene revealed that CSF-1R is encoded by 21 exons interspersed over 32 kilobases (kb) on the long arm of chromosome 5. Although transcription of c-*fms* mRNA in monocytes is initiated from a promoter located in close proximity to the CSF-1R initiator codon, transcripts in trophoblasts contain spliced 5′ noncoding sequences originating from an exon located 26 kb upstream. This upstream exon was located <0.5 kb from the 3′ end of the gene encoding the B-type platelet-derived growth factor receptor (PDGF-R). Based on the known intron-exon organization of c-*fms* and its sequence similarity to B-type PDGF-R, alignment of the two cDNAs

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enabled a correct prediction of splice junctions within the PDGF-R gene. Their overall homology and genome organization therefore suggest that CSF-1R and PDGF-R were derived from an ancestral gene that underwent duplication and subsequent divergence. Sequences governing CSF-1R expression in trophoblasts might reside within the PDGF-R gene, thereby providing a basis for their mutually exclusive expression in different cells of the placenta. A similar genomic organization might be expected for the A-type PDGF-R and c-*kit* genes on human chromosome 4.

III. Receptor Downregulation by CSF-1 and Transmodulation by Protein Kinase C.

In the absence of ligand, CSF-1R turns over with a half-life of 3–4 hours, but CSF-1 binding induces receptor internalization and degradation within minutes after stimulation. Receptor turnover is also accelerated after treatment of cells with agents such as phorbol esters that activate protein kinase C (PKC). The mechanisms of ligand- and PKC-induced degradation were shown to differ by several criteria. First, a kinase-defective receptor mutant containing a methionine-for-lysine substitution at its ATP-binding site was refractory to ligand but was rapidly degraded in response to phorbol esters. Second, in cells in which PKC was itself downmodulated by chronic phorbol ester treatment, receptors reexpressed at the cell surface remained sensitive to ligand-induced downregulation. Thus downregulation requires CSF-1R tyrosine kinase activity (but not PKC), whereas transmodulation depends on PKC (but not CSF-1R kinase) activity.

Phorbol ester treatment did not lead to receptor phosphorylation but rather induced proteolytic cleavage of CSF-1R near its transmembrane domain. This resulted in release of the CSF-1R ligand-binding domain from the cell and the transient appearance of a cell-associated 50 kDa fragment representing the tyrosine kinase domain. The latter polypeptide is probably inactive as an enzyme, because it lacks phosphotyrosine, the hallmark of the activated CSF-1R kinase. Thus physiologic agents that induce PKC in monocytes and macrophages should antagonize the CSF-1 response. Because CSF-1 functions as a survival factor for mature mononuclear phagocytes, transmodulation of CSF-1R on activated macrophages may serve to limit their life span during an inflammatory response.

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PROTEIN PHOSPHORYLATION IN NEURONAL FUNCTION

THOMAS R. SODERLING, Ph.D., *Investigator*

The major focus of the research in Dr. Soderling's laboratory this past year has been further elucidation of the regulatory mechanisms of Ca^{2+}/calmodulin-dependent protein kinase II (CaM-kinase II). This multifunctional protein kinase is highly abundant in brain, especially hippocampus, where it constitutes ~1–2% of total protein and 30–50% of the postsynaptic density protein. This kinase is thought to be involved in numerous neuronal functions, including regulation of neurotransmitter synthesis and exocytosis and perhaps in synaptic plasticity such as long-term potentiation.

The regulatory consequences of autophosphorylation of Thr-286 in the dodecameric holoenzyme were described last year: namely, conversion of the kinase to a partially Ca^{2+}-independent species. The essential role of Thr-286 has now been confirmed by mutation to either Ala or Asp. The Ala-286 mutant was just like the wild-type kinase, except that autophosphorylation did not generate a Ca^{2+}-independent form. The Asp-286 mutant kinase already exhibited Ca^{2+}-independent activity prior to autophosphorylation. This indicates that the presence of the negative charge at residue 286, either by phosphorylation of the Thr or by mutation to Asp, is sufficient for Ca^{2+}-independent activity.

The autoinhibitory domain in CaM–kinase II (residues 281–309) contains multiple regulatory elements. Residues 296–309 constitute the CaM-binding domain, residues 290–302 are inhibitory through interaction with the substrate protein-binding site, and residues 281–290 potentiate the inhibition by interfering with the ATP-binding site. The synthetic peptide containing residues 281–309 is a strong inhibitor of CaM-kinase II (K_i = 0.2 μM). The inhibitory potency of the synthetic peptide is completely reversed by binding of Ca^{2+}/CaM. Phosphorylation of Thr-286 greatly decreases its inhibitory potency but does not affect the binding of Ca^{2+}/CaM. On the other hand, phosphorylation of Thr-305 or Thr-306 prevents binding of Ca^{2+}/CaM.

These studies support the following model for the regulation of brain CaM–kinase II. In the inactive form, the autoinhibitory domain interacts with the catalytic binding sites for both protein and ATP. Binding of Ca^{2+}/CaM to residues 296–309 induces a conformational change in the overlapping inhibitory region (residues 281–302), thereby disrupting the interaction of the autoinhibitory domain with the catalytic site and activating the kinase. The activated kinase can phosphorylate exogenous protein substrates that contain the consensus recognition sequence (-R-R-X-S/T) as well as autophosphorylation on Thr-286. The autophosphorylated kinase is active in the absence of Ca^{2+}/CaM, since the negative charge at residue 286 prevents the autoinhibitory domain from interacting with the catalytic site. The Ca^{2+}-independent form undergoes further autophosphorylation on Thr-305–306 within the CaM-binding domain, which prevents binding of Ca^{2+}/CaM. This complex regulatory mechanism for CaM–kinase II presumably allows it to perform multiple modulatory roles in neuronal functions.

Dr. Soderling is Professor of Molecular Physiology and Biophysics at the Vanderbilt University School of Medicine.

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DEVELOPMENTAL CONTROL OF CHROMOSOME FUNCTION

ALLAN SPRADLING, PH.D., *Investigator*

The genetic information for development is stored on chromosomes. Each generation this information must be accurately copied, segregated to daughter cells, and recombined within the germ cells. Dr. Spradling's laboratory is interested in the molecular mechanisms involved in these chromosomal functions and how they relate to the actual use of developmental information through regulated gene expression.

I. A Model Chromosome.

Dr. Spradling's group has selected as a model the smallest known *Drosophila* chromosome, Dp1187. This minichromosome was produced (in the 1950s) by deleting 97% of the internal sequences from a normal X chromosome. Although just 10 genes from the X chromosome tip are appended to the basal 10% of the centromeric heterochromatin in Dp1187, it replicates and is transmitted with high fidelity. Pulsed-field gel electrophoresis revealed that Dp1187 contains only ~1.4 million base pairs (bp) of DNA, far less than any other known chromosome in a multicellular animal. The distal 300–400 kb encodes the known genes on the minichromosome, while the remaining 1 million bp represents heterochromatin, the little-understood chromosome domain associated in most higher organisms with centromeres.

A restriction map was constructed using pulsed-field gel electrophoresis of most of the gene-containing portion of the minichromosome and ~100 kb of the adjacent heterochromatin. This map provided sufficient information to analyze a long-standing problem in chromosome replication. Many *Drosophila* cells, such as the larval salivary gland, undergo polytenization, a specialized type of chromosome replication that sometimes produces giant polytene chromosomes. During polytenization the heterochromatic chromosome regions replicate much less than the remainder of the chromosome. As expected, Dp1187 heterochromatin was found to replicate less than the distal euchromatin in the salivary gland. Replication of the euchromatin depended on its distance from the junction with heterochromatin; the copy number of sequences increased in a gradient over at least a 100 kb region. Surprisingly, however, the replication of Dp1187 also varied from cell to cell within the salivary gland, and probably in many other adult tissues. These studies suggested that replication of heterochromatin may not always be as tightly controlled as the coding part of chromosomes.

These observations also provided insights into the phenomenon of position-effect variegation. Genes that are relocated next to a heterochromatic region as the result of a chromosome rearrangement frequently are expressed only in some of the cells within a tissue. The expressing cells are scattered, apparently at random, throughout the affected tissues. Genetic evidence shows that this is a long-range effect that spreads from the heterochromatic breakpoint into the adjoining euchromatin. The construction of Dp1187 created such a euchromatin-heterochromatin junction. Several genes on Dp1187, including *yellow*, show typical position-effect variegation in flies with the replication defects that were measured. Addition of a Y chromosome suppressed variegated *yellow* expression. The added Y chromosome also greatly increased the replication of Dp1187 euchromatin (including the *yellow* locus) in the salivary gland. Variable replication of Dp1187 therefore appears to be the underlying cause of the variegated expression of genes located near its euchromatin-heterochromatin junction.

II. Oogenesis.

Dr. Spradling's group has initiated genetic studies of several aspects of *Drosophila* oogenesis. The technique of single–P element insertional mutagenesis was used to generate and screen 5,000 lines for female sterile mutations. The expression patterns of an enhancer-sensitive *lacZ*-fusion gene residing on a mobilized P element were also studied within ovaries from an additional 9,000 lines. These studies have allowed Dr. Spradling's group to identify candidate genes involved in several processes in oogenesis, including stem cell maintenance, oocyte determination, follicle cell specialization, transmission of material between nurse cells and oocyte, and follicle cell–germ cell communication. A significant current task is to analyze the molecular processes giving rise to the common mutant phenotypes so that mutations involved in specific processes can be identified.

Studies of the *bag-of-marbles* (*bgm*) gene revealed a possible connection between sex determination and the ability of a germ cell to become an oocyte. Mutants at *bgm* are female sterile and lack

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developing eggs. As in the case of other tumorous ovary mutations, small proliferating cells fill the germarial region of each ovariole. A group of such cells is occasionally surrounded by follicle cells to form an abnormal cyst, which does not differentiate further. This same phenotype results when the major gene controlling somatic sex determination, *Sex-lethal*, cannot function in the germline. Female germline cells may require the action of *Sex-lethal* as well as tumorous ovary genes to prevent the development of germline cells along male pathways. Consistent with this model, morphological similarities were noted between the proliferating cells in *bgm* ovaries and normal spermatocytes. The *stellate* gene family, a locus normally expressed only in the testis, was active in *bgm* females. The *bgm* gene was cloned, rescued by transformation, and the structure of a major transcript characterized. The amino terminus showed limited homology to the gene *ovary tumorous*.

Dr. Spradling is also Staff Member in the Department of Embryology at The Carnegie Institution of Washington and Adjunct Professor in the Departments of Microbiology and Biology at The Johns Hopkins University.

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BIOSYNTHESIS AND ACTIONS OF ISLET HORMONES

DONALD F. STEINER, M.D., *Senior Investigator*

Progress during the past year includes studies on 1) basic cellular mechanisms of insulin production and action, 2) islet cell secretory products and their precursors, 3) mutations in insulin and insulin receptor genes in humans with diabetes or other metabolic syndromes, and 4) the structure and function of the insulin receptor.

I. Biosynthesis of Islet Hormones.

A. *Prohormone-converting enzymes.* Early studies from Dr. Steiner's laboratory, as well as others, have established that prohormone processing usually occurs via cleavage at pairs of basic residues, such as Lys-Arg or Arg-Arg, by a trypsin-like endoprotease. A carboxypeptidase B–like exopeptidase then excises the basic residues from the newly created carboxyl termini. Processing occurs mainly in secretory vesicles and also includes carboxyl-terminal amidation of some peptides. The nature of the mammalian processing endoprotease(s) has been controversial. A number of candidate enzymes have been proposed, but few, if any, of these have been shown to participate *in vivo*, except for the Kex2 enzyme of yeast, which cleaves the α-factor precursor.

Several years ago a project was begun to identify cDNAs from animal tissues that would complement the *KEX2* mutation in yeast. Direct screening of islet libraries yielded no positives. Vectors were then constructed for expressing islet cDNA libraries in yeast, and suitable Kex2-minus strains of yeast were developed. These efforts also failed to produce any positives. A new approach was then explored that involved the use of polymerase chain reaction (PCR) to search for sequences related to those of Kex2 in insulinoma mRNA. This approach has proven successful, and a cDNA that appears to be related to Kex2 from human insulinoma mRNA has recently been identified. The encoded protein retains the necessary catalytic residues for proteolysis, suggesting that it is an enzyme related to Kex2 and/or subtilisin. This full-length cDNA is currently being expressed in various animal cells, as well as in yeast and *Xenopus* oocytes, to explore the nature of the proteolytic activity that it may encode. A membrane-bound form of carboxypeptidase B has also recently been cloned in collaboration with Dr. Randal Skidgel (University of Illinois).

B. *Sorting mechanisms of beta cells.* Several years ago studies were initiated on a mutant proinsulin gene associated with familial hyperproinsulinemia. A single Asp for His substitution at position 10 of the B chain was found. Synthesis of the corresponding mutant insulin molecule by Dr. Panayotis Katsoyannis and co-workers revealed a binding and biological potency four- to fivefold greater than that of normal human insulin. A series of biosynthetic studies was then begun on islets from transgenic mice harboring this mutant human gene [in collaboration with Dr. Robert E. Hammer (HHMI, University of Texas Southwestern Medical Center at Dallas)]. The secretion of unprocessed mutant proinsulin into the medium is about five- to sixfold greater than that of the normal mouse prohormones. A significant fraction of the mutant human proinsulin appears to enter an unregulated or constitutive pathway. Further studies are in progress to assess the possible role of the elevated receptor-binding affinity of the mutant proinsulin in its aberrant sorting behavior.

II. Islet Amyloid Polypeptide (IAPP).

Recent studies have shown that amyloid deposits present in the islets of type II diabetics consist of a neuropeptide structurally related to calcitonin gene-related peptide (CGRP). Several laboratories reported amino acid sequences for this 37–amino acid peptide (also called amylin) from diabetic human as well as cat pancreas. Studies by Dr. Per Westermark and his associates in Uppsala have shown that this peptide is present in the beta cells and is stored in the insulin granules. To gain a better understanding of its biosynthesis, Dr. Steiner and his colleagues isolated a full-length cDNA encoding the IAPP precursor from a human insulinoma library. The predicted amino acid sequence revealed a polypeptide of 89 amino acids, beginning with a typical signal peptide followed by a prohormone containing the IAPP moiety bracketed at each end by pairs of basic residues. A glycine residue just beyond the carboxyl terminus of IAPP indicated that this peptide would likely be amidated during its processing in the beta cell. Further comparative studies in cats, rats, mice, and guinea pigs have shown that the IAPP sequence is highly conserved, while the remainder of the precursor shows a much higher level of variation, sug-

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gesting that only the IAPP portion is hormonally active. The chromosomal gene that encodes IAPP is located in the p12.3 region of chromosome 12. Studies are under way to determine whether insulin and IAPP are synthesized and released in a coordinate fashion and whether there may be differences in the expression of IAPP and insulin under certain conditions.

III. Insulin Receptor Expression and Maturation.

To generate sufficient amounts of human insulin receptor-related materials for studies of its three-dimensional structure and insulin-binding region, Dr. Steiner and his colleagues explored several systems for the overproduction of the receptor. One of these, the baculovirus system, utilizes insect cells and viral vectors to overproduce proteins of interest. Suitable vectors have been constructed for the expression of the insulin holoreceptor, as well as truncated versions in which the transmembrane and internal regions have been deleted from the β-subunit so that a soluble form of the receptor is secreted from the cells. Very high levels of expression of normal and mutated insulin receptors have also been obtained using dihydrofolate reductase (DHFR)-vector systems that are suitable for expression in animal cells such as the CHO (Chinese hamster ovary) cell line. Among receptor mutants being studied using these systems are several involving the proteolytic cleavage site of the α/β-proreceptor in which the tetrabasic cleavage recognition sequence has been systematically modified by the replacement of individual basic residues with alanines. Recent studies from this laboratory have indicated that the insulin-binding region in the receptor is located near the amino terminus of the α-subunit, probably somewhere within the first 100 amino acids.

IV. Evolution of Insulin and Insulin Receptor Molecules.

The evolution of insulin and insulin-like growth factors (IGFs) and the coevolution of the insulin and IGF receptor molecules are currently being explored in a variety of vertebrate and/or nonvertebrate species. Several cDNAs encoding IGF-like molecules from both the coho salmon (a teleost fish) and the hagfish (a jawless vertebrate) have now been identified. The salmon IGF-I–like peptide appears to be under growth hormone control. These studies will be extended to protochordate species such as *Amphioxus*. The conservation of features in insulin and IGF receptors that are believed to be important for binding of insulin or IGF in various vertebrate species is also being studied, using PCR methods to amplify appropriate regions.

Dr. Steiner is also the A. N. Pritzker Distinguished Service Professor of Biochemistry and Molecular Biology and of Medicine at The University of Chicago.

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CELL BIOLOGY OF EFFECTOR PROTEINS

LINDA J. VAN ELDIK, PH.D., *Associate Investigator*

One of the long-term goals of the research in Dr. Van Eldik's laboratory is to increase understanding of the molecular mechanisms controlling the pathways by which the brain develops from a single cell into a complex organ capable of functions such as learning, memory, motor control, and perception. A growing body of evidence suggests that an important aspect of regulation of neuronal development and maintenance involves soluble neurotrophic factors, i.e., molecules that enhance neuronal survival and/or stimulate neurite outgrowth. The prototypical neurotrophic factor is nerve growth factor, whose effects include maintenance of the differentiated state of mature sympathetic and sensory neurons, enhancement of neuronal survival, and promotion and guidance of neurite outgrowth.

Dr. Van Eldik's laboratory has recently shown that a form of S100β, a protein found in high levels in glial cells, has neurotrophic activity on central nervous system (CNS) neurons, specifically on neurons of the cerebral cortex and spinal cord. S100β belongs to a family of proteins with primary sequence similarities. The S100 family includes S100α and S100β; calpactin light chain; S100L; proteins that are elevated in patients with cystic fibrosis (CF) and rheumatoid arthritis (CF antigen, MRP-8, and MRP-14); and predicted protein sequences deduced from RNAs that are expressed in high levels in differentiated, transformed, or growth factor–treated cells (calcyclin, 18A2, 42A and 42C, p9Ka, pEL98). The observation that expression of members of the S100 family is altered during cell growth, differentiation, and in certain diseases suggests that they may play regulatory roles in these processes.

Although little information is available about the *in vivo* roles of many of the members of the S100 family, a disulfide form of S100β has been shown to stimulate neurite outgrowth in primary cultures of cortical neurons, in a neuroblastoma cell line, and in organotypic cultures of spinal cord/ganglia. The form of S100β with neurotrophic activity is referred to here as NEF (neurite extension factor). The observation that NEF has neurotrophic activity *in vitro*, coupled with the presence of extracellular S100 in brain and glial cell cultures, suggests that during development of the nervous system, NEF might be released from glial cells and act in a paracrine fashion to stimulate neurite outgrowth. The potential importance of this protein as a neurotrophic factor is also indicated by its localization in the developing nervous system during the time of elongation of neuronal processes. Altogether, the data suggest that NEF may be a neurotrophic factor synthesized and released by glial cells for neurons of the CNS.

To develop the necessary reagents for addressing the long-term question of how NEF may be involved in neuronal development and maintenance, Dr. Van Eldik's laboratory previously synthesized a gene coding for S100β, expressed the gene in *Escherichia coli*, and produced protein (VUSB-1) by recombinant DNA technology. VUSB-1 preparations have neurotrophic activity on embryonic chick cortical neurons, whereas preparations of *E. coli* transformed with vector lacking the S100β gene do not have activity. VUSB-1 enhanced cell maintenance in culture as well as stimulated neurite extension. In addition, in control experiments, the neurite extension activity was reduced by preincubation with antibodies made against bovine brain S100β. Finally, VUSB-1 preparations lose activity when treated with reducing agents or when purifications are done in the presence of reducing agents; this is consistent with the idea that the activity of NEF requires a disulfide form of the protein. This apparent requirement for a disulfide linkage in order to function as a neurotrophic factor is intriguing in light of the fact that S100β proteins isolated from different species and tissues have invariant cysteines at residues 68 and 84. In addition, S100β is the only member of the S100 family of proteins that has cysteines at both of these positions in the amino acid sequence; all other members have a valine at the position analogous to Cys-68.

Dr. Van Eldik's recent studies using site-directed mutagenesis/protein engineering approaches have shown that both cysteine residues of VUSB-1 are important for neurite extension and cell survival activity but that the relative position of the two cysteines can be altered without loss of activity. Specifically, the relative contribution of Cys-68 and Cys-84 to the NEF activity of VUSB-1 was tested by preparing mutant VUSB-1 proteins with Cys-68 changed to alanine, serine, or valine; Cys-84 was changed to alanine or serine; or both cysteines were changed (Cys-68 to valine and Cys-84 to alanine or serine). Mutant proteins lacking either Cys-68 or Cys-84 were unable to stimulate neurite extension or enhance neuronal survival. These data indicate that

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the linear amino acid sequence alone is not sufficient for neurotrophic activity but that secondary structural features, i.e., disulfide bonds, are important for both neurite extension and neuronal survival activities. In addition, the fact that both cysteine residues are necessary for biological activity suggests an involvement of both residues in the disulfide linkage of the active species of the protein. An analysis of the secondary structural constraints on the protein for activity was begun by changing the relative position of the two cysteines in the linear amino acid sequence. A mutant was prepared with Ser-62 changed to cysteine and Cys-68 changed to valine. The mutant still has two cysteine residues, but the cysteine that is normally found at residue 68 has been moved to residue 62. This mutant stimulated neurite extension and enhanced cell survival in a manner similar to the unmodified VUSB-1. These data suggest that the structural requirements for neurotrophic activity are somewhat flexible; the relative position of the two cysteine residues can be altered without significantly diminishing the neurotrophic activity.

This flexibility in the relative positioning of the cysteine residues in the linear sequence has important pharmacological implications. The ability to design synthetic neurotrophic agents based on the S100β structure has the potential to provide a rational approach to the development of reagents useful for nerve regeneration or selective maintenance of neuronal function. In addition, the data support the hypothesis that a disulfide-linked form of S100β may act as a neurotrophic factor in the CNS, provide a foundation for future studies into the effects of NEF *in vivo* during CNS development, and offer insights into the molecular regulation of neuronal development and maintenance.

Dr. Van Eldik is Associate Professor of Pharmacology and Cell Biology at Vanderbilt University School of Medicine.

PUBLICATIONS

Article

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MOLECULAR PHYSIOLOGY OF CALCIUM

D. MARTIN WATTERSON, PH.D., *Investigator*

Eukaryotic cells respond to a diversity of physiological and pharmacological stimuli by molecular mechanisms that include transient rises in the intracellular concentration of ionized calcium. These intracellular calcium signals are transduced into biological responses through the action of a class of calcium-binding proteins that includes calmodulin (CaM). Although there is a family of highly similar calcium-binding proteins in this class (e.g., troponin C from skeletal muscle tissue has a number of physical and chemical similarities to CaM), many of these other calcium-binding proteins are more restricted in their tissue or phylogenetic distribution. CaM is ubiquitous among eukaryotes and has multiple biological roles. Therefore it has been used as a prototypical example of this class of calcium-binding proteins.

CaM is an integral regulatory subunit of several enzymes, cytoskeletal structures, and membrane transport systems. An intriguing situation that appears to be unique to CaM is the fact that a single eukaryotic cell can contain several of these CaM:enzyme signal transduction complexes, and a particular CaM:enzyme complex can be found in multiple tissues and cell types. Therefore, to understand how a eukaryotic cell is able to manage calcium signals in a selective and quantitative manner, it is necessary to have a detailed understanding of the mechanism of action of this calcium signal transducer, which appears to be fundamental to life and eukaryotic cell homeostasis.

The goal of Dr. Watterson's laboratory is to understand how CaM is able to transduce calcium signals in eukaryotic cells into a specific set of biological responses and integrate these responses with other signal transduction pathways. Significant advances have been realized during the past year as a result of the culmination of studies employing protein engineering and site-specific mutagenesis, approaches first applied to this field of research by Dr. Watterson's laboratory. Detailed analyses of CaM and one CaM:enzyme complex and selected studies of other CaM-regulated enzymes have provided a generalized model of how CaM transduces a calcium signal into a biological response. This model proposes that there is a dynamic equilibrium among calcium, CaM, enzyme, and substrate, with the transient rise in ionized calcium that occurs with a cellular stimulus resulting in a perturbation of the equilibrium. Based on this model and an increased knowledge of the structural basis of CaM recognition by the enzyme, a rational basis for the design and production of new chimeric proteins has emerged, as well as a molecular model of how inherited mutations of genes encoding CaM or a CaM-regulated enzyme might bring about a selective, nonlethal pathology.

The group of CaM-regulated enzymes that served as a focal point were the protein kinases that have CaM as a regulatory subunit. These enzymes include the phosphorylase kinases, the myosin light chain kinases (MLCK), and the type-II CaM-dependent protein kinases (CaMPK-II). Recently Dr. Watterson's laboratory demonstrated, through the combined use of computational chemistry, site-specific mutagenesis, and enzyme regulatory activity analyses of CaM, that, in addition to hydrophobic interactions, certain charge features in both halves of the CaM molecule are critically important for the activation of CaM-regulated protein kinases. Furthermore, these charge properties of CaM appear to provide an element of selectivity to CaM's interaction with various enzymes, especially among protein kinases. For example, there is a selective preference by MLCK for the presence of a carboxylate function at residues 84 and 120 in two different α-helices of CaM that flank a recessed hydrophobic surface in the carboxyl-terminal half of CaM. In contrast, the activity of CaM:CaMPK-II complexes is slightly perturbed by changes at residue 120 of CaM (carboxyl-terminal domain) but not by changes at residue 84 of CaM (central helix). Consistent with the differences in regulatory activity, the ability of MLCK or CaMPK-II to bind CaM was selectively perturbed by mutations in these regions of CaM.

The approach in these studies was based on the concept of perturbation mutagenesis screening and chemical complementarity in macromolecular recognition. The key observations that formed the foundation of the studies were 1) the presence in CaM of amphiphilic helices with an asymmetric distribution of negative-charge properties, 2) the ability to perturb selectively the activity of CaM:enzyme complexes when helical regions of CaM are perturbed by charge-reversal site-specific mutagenesis, and 3) the presence of positive-charge clusters in peptide fragments of MLCK that bind CaM with an affinity and selectivity approximating that of the native enzyme. The distance between Glu-84 and Glu-

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120 of CaM could, theoretically, be spanned by the 20-residue CaM-binding region of MLCK (RS20), an amino acid sequence first shown by Dr. Watterson's laboratory to be a high-affinity, CaM-selective binding fragment of smooth muscle MLCK. The RS20 CaM-binding segment appears to be in an α-helical conformation when bound to CaM and contains a potentially complementary basic-hydrophobic amino acid sequence motif. Similar primary and secondary structural motifs were detected in other CaM-regulated protein kinases, and synthetic peptide analogues of portions of these sequences were shown to bind CaM with high affinity and selectivity.

Protein engineering and site-specific mutagenesis, based on pattern analysis and perturbation of chemical complementarities in macromolecular interactions, were used to identify a CaM recognition/intramolecular signal transduction region within a CaM-regulated protein kinase. These results with the protein kinase were based on four interrelated sets of investigations: 1) the design and production in *Escherichia coli* of a CaM-dependent enzyme indistinguishable from the tissue-isolated enzyme in its kinetic and CaM regulatory properties, 2) identification by deletion mutagenesis of the approximate boundaries of the CaM regulatory region, 3) the design and production of chimeric enzymes with the catalytic properties of an MLCK but the CaM recognition/regulatory properties of either an MLCK or a CaMPK-II (dependent on which CaM recognition sequence was inserted), and 4) the design and production of a suppressor mutation in the RS20 CaM-binding segment of the protein kinase that restores toward normal the functional effects of point mutations in CaM.

In summary, the results of computational, site-specific mutagenesis and time-resolved spectroscopic studies of CaM suggest that CaM exists in a series of multiple, interconverting conformations in solution, with the addition of calcium and RS20 peptide analogues resulting in restrictions on the population of conformations and their rate of interconversion. Recent structural data from other laboratories for CaM:peptide complexes, in which CaM-binding peptides from skeletal muscle MLCK were studied by nuclear magnetic resonance and neutron-scattering techniques, are consistent with this model. If the existence of such a multicomponent dynamic equilibrium is assumed, the effect of calcium and protein-protein contact may be to perturb this dynamic state by restriction of the population of conformations, i.e., selection of preferred conformations by the two proteins. The results of the past year have enhanced the understanding of which features of CaM and the enzyme are important in coupling these intermolecular interactions (intermolecular signaling) to an effective perturbation of intramolecular interactions within the enzyme (intramolecular signaling), with a resultant catalytically productive interaction between enzyme and substrates.

Dr. Watterson is also Professor of Pharmacology at Vanderbilt University School of Medicine.

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MOLECULAR BIOLOGY OF GROWTH FACTOR RECEPTOR SIGNAL TRANSDUCTION

LEWIS T. WILLIAMS, M.D., PH.D., *Investigator*

Experiments performed in Dr. Williams's laboratory this year provided insight into the mechanism by which the platelet-derived growth factor (PDGF) receptor functions in signal transduction. Although most of these studies focused on the PDGF β-receptor, preliminary experiments with the closely related PDGF α-receptor suggest that it functions in a similar manner. The data suggest a model in which the binding of PDGF to the extracellular domain stimulates the following sequence of events: the receptor forms a dimer that becomes autophosphorylated; the conformation of the cytoplasmic domain is altered by autophosphorylation; the receptor then physically associates with at least three, and possibly four, cytoplasmic signaling molecules; the receptor tyrosine kinase phosphorylates the signaling molecules on tyrosine residues. Experiments done in Dr. Williams's laboratory by Dr. Deborah Morrison showed for the first time that the biological activity of one of the signaling molecules, the Raf-1 protein, was altered by the direct phosphorylation of the protein by the receptor. Thus tyrosine phosphorylation of signaling molecules may regulate their activities, and the physical association of these molecules with the receptor may localize their activities.

I. PDGF-induced Receptor Association and Tyrosine Phosphorylation of Signaling Molecules.

For the last three years Dr. Williams's group has attempted to identify the molecules that associate with the ligand-activated PDGF receptor and serve as substrates of its tyrosine kinase. Their first progress occurred last year, when Drs. Shaun R. Coughlin (HHMI, University of California at San Francisco) and Jaime Escobedo showed that a 3′-phosphatidylinositol kinase was physically associated with the PDGF-stimulated receptor. This work was extended this year to the study of other signaling molecules.

A. *Raf-1.* The Raf-1 protein is a 74 kDa serine/threonine kinase that has an oncogenic counterpart described previously by Dr. Ulf Rapp at the National Institutes of Health. Dr. Morrison showed that the Raf-1 protein coimmunoprecipitated with the PDGF receptor when extracts of PDGF-stimulated cells were precipitated with either antireceptor or anti-Raf antibodies. Extracts of unstimulated cells showed no association of receptor and Raf-1 protein. Immobilized, highly purified autophosphorylated PDGF β-receptor (produced by Dr. Escobedo using a baculovirus expression system) was used to adsorb the PDGF receptor from 3T3 cell extracts *in vitro*. Dr. Morrison also showed that when the receptor was dephosphorylated by phosphatase treatment it lost its ability to bind to Raf-1, suggesting that only the phosphorylated form of the receptor was capable of interacting with the Raf-1 protein. In other experiments, insect cells were co-infected with recombinant PDGF receptor baculovirus (constructed by Dr. Escobedo) and recombinant Raf-1 baculovirus (constructed in the laboratory of Dr. Thomas Roberts, using the Raf-1 cDNA provided by Dr. Rapp). The Raf-1 protein in these cells became phosphorylated on tyrosine in response to PDGF, and the receptor was physically associated with the Raf-1 protein. In more direct *in vitro* experiments, highly purified PDGF β-receptor protein was incubated directly with highly purified Raf-1 protein. In the presence of ATP the receptor directly phosphorylated the Raf protein on tyrosines and caused an increase in Raf-1 kinase activity of four- to sixfold *in vitro*.

A mutant PDGF β-receptor protein that had a large deletion of the domain that interrupts the tyrosine kinase–coding sequence was also expressed by Dr. Escobedo and Sutip Navankasattusas and was immunopurified for *in vitro* experiments. This kinase insert (KI) deletion mutant had the same autophosphorylating activity as the wild-type β-receptor but failed to phosphorylate the Raf-1 protein and failed to activate Raf kinase *in vitro*. When expressed in intact cells the KI deletion mutant receptor was defective in its phosphorylation of Raf-1 kinase and stimulation of DNA synthesis. These experiments show that the Raf-1 protein is a substrate of the PDGF receptor kinase and that regulation of the Raf kinase activity is an important step in PDGF-induced mitogenesis.

B. *Phospholipase C-γ.* Working in Dr. Williams's laboratory, Dr. David Kaplan showed that phospholipase C-γ, an enzyme that hydrolyzes phosphatidylinositol to produce diacylglycerol and inositol phosphate second messengers, associates with ligand-activated PDGF receptor in intact cells and with highly purified and autophosphorylated receptor *in vitro*. Phospholipase C-γ, in contrast to

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the Raf-1 protein, was readily phosphorylated by the KI deletion mutant of the receptor, both in intact cells and *in vitro*. This is consistent with the ability of this mutant to stimulate PI hydrolysis in intact cells.

C. *3'-Phosphatidylinositol kinase (PI kinase).* This enzyme, which phosphorylates phosphatidylinositol at the 3 position of the inositol ring, was the first protein shown to be associated directly with the ligand-activated PDGF receptor in intact cells. This year, Dr. Kaplan showed that purified and immobilized PDGF receptor bound to PI kinase in 3T3 cells *in vitro*.

Together these experiments show that ligand-activated PDGF receptor physically associates with several transducing molecules and phosphorylates the molecules on tyrosine residues. Preliminary experiments suggest that the receptor domains that are involved in these protein associations are distinct for each signaling molecule. Physical association of the receptor with the signal-transducing molecules may be involved in localizing the activities of these enzymes. The experiments on the Raf kinase provide the first direct evidence of a biological activity that is directly altered by a receptor tyrosine kinase.

II. Autophosphorylation Sites of the PDGF Receptor.

Dr. Escobedo and Dr. Wendy Fantl showed that substitution of phenylalanine for tyrosine at position 825 of the mouse PDGF β-receptor caused a critical alteration in the substrate specificity of the receptor's tyrosine kinase and a concomitant deficiency in the ability of the receptor to mediate PDGF-stimulated DNA synthesis. The roles of this and other PDGF receptor autophosphorylation sites in ligand-mediated association of the receptor with signaling molecules is currently under investigation.

III. Fibroblast Growth Factor Receptor.

The fibroblast growth factor (FGF) receptor was purified from 50,000 chicken embryos by Dr. Pauline Lee in Dr. Williams's laboratory. Amino acid sequences of tryptic digest fragments of the receptor were obtained by Dr. Victor Fried (St. Jude Children's Research Hospital). Dr. Lee and Dr. Daniel Johnson cloned the cDNA for the chicken FGF receptor and for the human analogues of the receptor. The chicken FGF receptor, expressed in *Xenopus* oocytes by microinjection of mRNA, mediated FGF-stimulated changes in calcium flux. Ongoing work on the role of this receptor and the developmental biology in angiogenesis will be supported in the future by the National Institutes of Health; HHMI will support work on the structure of this receptor.

Dr. Williams is also Professor of Medicine at the University of California at San Francisco.

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SOMATIC GENE TRANSFER IN THE STUDY AND TREATMENT OF METABOLIC DISEASES

JAMES M. WILSON, M.D., PH.D., *Assistant Investigator*

Research in Dr. Wilson's laboratory focused on the use of somatic cell gene transfer in the study and treatment of metabolic diseases. A variety of somatic cells were used as targets for gene transfer, including hepatocytes, vascular endothelial cells, and hematopoietic stem cells.

I. Liver-directed Gene Transfer.

Familial hypercholesterolemia (FH) is an autosomal dominant disorder in humans caused by deficiency of the receptor for low-density lipoproteins (LDL) that Dr. Wilson has used as a model for developing liver-directed gene therapies. This genetic disease is a useful model because 1) biochemical, pathophysiological, and clinical aspects of FH have been well described, and the corresponding normal gene is available; 2) phenotypic correction of the metabolic abnormalities associated with FH will probably require gene transfer into the hepatocyte, because the liver is the primary organ responsible for degradation of LDL and the only organ capable of excreting cholesterol; 3) an authentic animal model for FH exists, the Watanabe heritable hyperlipidemic (WHHL) rabbit; and 4) no effective conventional therapy exists for homozygous deficient FH, other than combined liver heart transplantation.

One approach to the genetic treatment of FH is similar in concept to the well-described bone marrow gene therapies. This method involves isolating hepatocytes from a genetically deficient animal, transferring a functional LDL receptor gene to hepatocytes *in vitro*, and transplanting the genetically modified cells back into the affected animal. Hepatocytes were harvested from WHHL rabbits, plated in primary cultures, and exposed to recombinant retroviruses capable of efficiently transferring a functional LDL receptor gene. The genetically modified cells were harvested and infused into the portal vein of WHHL recipients; serial measurements of serum cholesterol were performed. Similar transplantation experiments were performed with the hepatocytes derived from an allogeneic strain of rabbits that expresses normal levels of LDL receptor. In each case, transplantation of hepatocytes that express LDL receptor into WHHL rabbits consistently led to substantial decreases in total serum cholesterol for up to 14 days; fractionation of lipoproteins indicated that this is largely due to diminished LDL cholesterol.

A potentially more effective and less morbid approach to the genetic treatment of FH is to target the delivery of a functional LDL receptor gene to hepatocytes *in vivo*. Dr. Wilson is collaborating with Drs. George and Cathy Wu (University of Connecticut) to develop methods for targeting recombinant genes to hepatocytes that are based on interactions with the hepatocyte-specific receptor, the asialoglycoprotein receptor. The most encouraging results have been obtained with a synthetic DNA-protein complex. A high-affinity ligand for the asialoglycoprotein receptor (asialoorosomucoid) is covalently linked to polylysine, and this protein conjugate is coupled with DNA of an expression vector. When injected into the venous circulation of rats, the protein-DNA complex is specifically internalized by hepatocytes via the asialoglycoprotein receptor, and the reporter gene of the vector is expressed for ~2–3 days. Expression has been demonstrated in rat liver *in vivo* with vectors that contain constitutive as well as liver-specific transcriptional elements. In an attempt to achieve long-term expression of the recombinant gene, rats were subjected to partial hepatectomy immediately after the administration of DNA-protein complex. High-level expression of the reporter gene has been detected in livers of these animals for at least 4 months. The mechanisms responsible for this persistent transgene expression have not been defined. Similar approaches will be used to target the delivery of functional LDL receptor genes to the livers of WHHL rabbits.

II. Recombinant Gene Expression in Vascular Endothelial Cells.

The endothelial cell is an important component of blood vessels that is involved in the maintenance of vascular homeostasis. Dr. Wilson, in collaboration with Dr. Richard Mulligan (Whitehead Institute), has explored the possibility of using the vascular endothelial cells to target for gene transfer.

Replication-defective retroviruses were used to transduce recombinant genes efficiently into primary cultures of endothelial cells established from human, canine, and bovine vascular tissues. A variety of recombinant genes have been expressed in endothelial cells using this system, including those that encode for platelet-derived growth factor re-

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ceptor, LDL receptor, parathyroid hormone, adenosine deaminase, and β-galactosidase from *Escherichia coli* (i.e., *lacZ*). Transplantation of genetically modified endothelial cells was demonstrated in a canine model previously used to study thrombosis. Recombinant retroviruses were used to transduce the *lacZ* gene into canine endothelial cells. Prosthetic vascular grafts seeded with genetically modified cells were implanted as carotid interposition grafts into the dogs from which the original cells were harvested. Analysis of the grafts 5 weeks after implantation revealed genetically modified endothelial cells lining their luminal surfaces. This technology provides an opportunity to study the effects of recombinant gene expression on endothelial cell function *in vivo* and has potential applications to the treatment of vascular disease and the design of new drug delivery systems.

Dr. Wilson is also Assistant Professor of Internal Medicine and of Biological Chemistry at the University of Michigan Medical School.

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II. PROGRAM IN GENETICS

In recent years genetics has come to occupy a critical position in all biomedical research. It is not surprising, therefore, that it should be the largest of the Institute's research programs. Much of the work being carried out in the Institute's other programs also depends to a greater or lesser extent on the techniques and conceptual underpinnings of genetics in general and molecular genetics in particular. Investigators who are specifically associated with the Program in Genetics are located at the University of Michigan, The Johns Hopkins University, The Carnegie Institution of Washington at Baltimore, Harvard Medical School, Children's Hospital in Boston, the University of Colorado at Boulder, The University of Chicago, the University of Texas Southwestern Medical Center at Dallas, Duke University, Baylor College of Medicine, the University of Iowa, Yale University, The Rockefeller University, Stanford University, the California Institute of Technology, the University of Pennsylvania, Princeton University, the University of Utah, the University of Washington, Brandeis University, and the University of California at Berkeley, at Los Angeles, and at San Francisco. Collectively their work includes studies of human genetic diseases and various animal models of these diseases, analysis of developmental and differentiation processes, experimental studies of the regulation of gene expression, and the mapping of identified genes within the human genome and in the genomes of other animals. Their work ranges from basic studies at the molecular and cellular level through clinical studies of the human genetic disorders to the development of potential therapeutic approaches.

Understanding the processes that govern the development of a complex organism from a single fertilized egg is one of the central problems in modern biology. From the fertilized egg must arise many thousands of cell types, each with its unique properties and functions. Those functions are determined to a large extent by the subset of genes that the cell chooses to express, and it is the basis for those choices that concerns the laboratory of Investigator Shirley M. Tilghman, Ph.D. (Princeton University). Considerable progress has been made in the past several years in identifying the signals encoded by the DNA surrounding specific genes that determine in which cells those genes will be expressed. These genetic signals are complex, often present in multiple copies, and are presumed to be sites at which proteins interact with the DNA to either enhance or diminish the expression of the genes in question. In the case of the α-fetoprotein gene (a gene that is activated in three cell types in the developing mouse embryo), at least five different DNA signals have been identified that are necessary to activate the gene appropriately and repress its expression later in development. Dr. Tilghman's laboratory is currently focusing on the identification of the proteins that interact with the DNA signals, anticipating that these proteins and the genes that encode them are critical components in determining cell type-specific expression of genes.

The interest of Senior Investigator Philip Leder, M.D. (Harvard Medical School) is in understanding some of the genes that control cell growth and development. The research of his laboratory, which is particularly relevant to the cancer problem, is largely focused on genes that modify an organism's susceptibility to the development of malignancy. Recently Dr. Leder and his colleagues have shown how genes that perturb the maturation of certain types of cells can protect the organism from the development of cancer and that certain other gene products, lymphokines, can act as potent antitumor agents, mobilizing the organism's defenses against the establishment of malignant tumors. Still other studies in Dr. Leder's laboratory have identified genes that perturb embryonic development and can lead to serious birth defects.

Recently developed techniques of gene targeting (which involves homologous recombination between DNA sequences residing in the chromosome and newly introduced, cloned DNA sequences) have made it possible, in principle, to generate mice with any desired genotype. This technology, which was perfected by Investigator Mario R. Capecchi, Ph.D. (University of Utah) is now being used to dissect genetically the developmental program responsible for specifying organogenesis and morphogenesis of the mouse.

Investigator Richard D. Palmiter, Ph.D. (University of Washington) and his colleagues, in collaboration with Dr. Ralph Brinster (University of Pennsylvania), use a different technique that involves the transfer of selected genes into the germline of mice to study various aspects of development and disease. The genes of interest are microinjected into the pronuclei of fertilized eggs, where they often integrate into one of the endogenous chromosomes and are later expressed as new genetic traits.

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By combining regulatory regions of one gene with a structural gene coding for a particular protein, the expression of the structural gene can be regulated in novel ways. With this approach it has been possible for these researchers to demonstrate a number of interesting biological phenomena. These include 1) increasing the growth of mice by directing the expression of growth hormone to large organs such as the liver, 2) promoting tumorigenesis by directing the expression of oncogenes to specific cell types, 3) deleting specific cells by directing the expression of a toxin to those cells, 4) directly visualizing certain developmental processes by using a receptor gene that codes for an easily detectable product, 5) demonstrating the functions of certain genes suspected of being involved in disease processes, and 6) experimentally mimicking certain genetic diseases.

Assistant Investigator Philippe M. Soriano, Ph.D. (Baylor College of Medicine) and his colleagues are using retroviral mosaics and the consequences of certain insertional mutations in developmental genes to study cell lineages during early mouse development. They have constructed new retroviral vectors in which a reporter gene, coding for the bacterial enzyme β-galactosidase, is placed under the control of an internal promoter allowing expression of the gene in the early embryo. They are also generating transgenic mice that are screened for instances of insertional mutagenesis by infection of mouse embryos or embryonic stem (ES) cells. The technique of homologous recombination has been used in ES cells to achieve targeted mutagenesis of the gene encoding c-*src*. The resulting chimeras are now being tested for germline contribution.

The laboratory of Assistant Investigator Paul A. Overbeek, Ph.D. (Baylor College of Medicine) also makes use of transgenic mice to study mammalian development. Two of the transgenic families under study in his laboratory have developmental abnormalities caused by insertional mutations. One mutation causes a defect in hair follicle formation; the other results in a defect in sperm maturation. For both of these new strains, the genomic sequences flanking the transgenic inserts have been cloned and are being used to examine the inactivated genes. In addition to the insertional mutants, a crystallin promoter has been used to direct lens-specific expression in transgenic mice. Two different constructs have been studied in some detail: one encodes a truncated SV40 early region, which causes microphthalmia; the other codes for a growth factor that causes abnormal development of the iris of the eye.

Transgenic animals are also used in the laboratory of Assistant Investigator Robert E. Hammer, Ph.D. (University of Texas Southwestern Medical Center at Dallas) to investigate the regulation of eukaryotic gene expression and to examine the physiologic consequences of selected gene expression. Of special interest is the function of the human low-density lipoprotein (LDL) receptor in transgenic mice and rabbits. Fusion genes containing the LDL receptor under the direction of various promoter/enhancer elements, including the mouse transferrin or the human β_2-microglobulin promoter, have been expressed in mice, and lines of animals are being used to investigate such questions as the differences in LDL receptor internalization in various somatic tissues as well as the nature of receptor-mediated endocytosis of plasma lipoproteins by peritoneal macrophages. Transgenic mice containing and expressing a variant form of LDL, Lp(a), will be used to investigate the role of Lp(a) in the progression of atherosclerosis.

Embryonic development is controlled by a network of interacting regulatory genes. Many of the genes are active in some parts of the embryo but not in others, thus causing cells in different places to do different things. The regulatory genes coordinate cell division and migration as well as the production and localization of specialized products. The molecular mechanisms through which genes interact and the nature of the proteins that do the regulating are being studied in the fruit fly *Drosophila* by the laboratory of Associate Investigator Matthew P. Scott, Ph.D. (University of Colorado at Boulder). Some regulatory genes encode proteins that control the activation of banks of other genes in specific cells. Other genes encode proteins involved in communication among cells.

Many of the genes involved in the early development of *Drosophila* have been identified, and a significant number of these genes contain a homeodomain, a DNA-binding motif also present in the genomes of mammals. Assistant Investigator Claude Desplan, Ph.D. (The Rockefeller University) and his colleagues have asked how proteins with related sequences perform their specific roles during development. They have found that a single amino acid of the homeodomain determines its DNA-binding specificity and therefore its function. Another level of specificity is achieved by the role of the proteins on transcription. Thus the homeodomain protein Engrailed represses transcription *in vitro,* whereas

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the Fushi tarazu protein activates after binding to the same DNA sites. The function of another class of transcription factors encoded by the so-called gap genes has also been investigated by Dr. Desplan's laboratory. These zinc finger proteins act early in development to set up segmentation. Binding sites for the Krüppel and Hunchback proteins have been defined in the *hunchback* promoter, and their function is now being tested *in vivo*.

During the development of an organism, information for the determination of different cell types becomes unevenly distributed in cells and tissues. In *Drosophila* the transcript of the *bicoid (bcd)* gene is localized to the anterior end of the oocyte; this results in a concentration gradient of *bcd* protein in the developing embryo. The product of the gene *exuperantia (exu)* is required for localizing the *bcd* transcript and for the formation of normal sperm in male flies. The laboratory of Assistant Investigator Tulle Hazelrigg, Ph.D. (University of Utah) has cloned the *exu* gene and is studying how it functions in oogenesis to bring about the subcellular localization of the *bcd* RNA, and also in spermatogenesis. Patterns of gene expression within a given tissue can be influenced by regulatory sequences in the DNA flanking a gene. When a gene is moved to a new location in the genome, other regulatory sequences can cause its expression in novel patterns. For example, the DNA flanking a relocated copy of the *white* gene causes a restriction in its expression to certain cells in the eye. Analysis of this flanking DNA by Dr. Hazelrigg's laboratory has revealed regulatory sequences that appear to act in conjunction with regulatory sequences within the promoter of the *white* gene to bring about this pattern of expression.

The laboratory of Assistant Investigator Norbert Perrimon, Ph.D. (Harvard Medical School) is studying the molecular biology of the genes involved in pattern formation. Two developmental systems are under investigation: 1) the genes involved in the determination of the antero-posterior axis intrasegmental patterning of the organism and 2) genes involved in the determination of a small number of cells in the central nervous system. This laboratory is also developing techniques for the ectopic activation of genes in order to manipulate the expression of the gene products involved in pattern formation and to contribute to the growing understanding of how developmental genes act.

Two homeotic genes, *abd-A* and *Abd-B*, that dictate the fate of cells in the abdominal segments of *Drosophila melanogaster* are under study in the laboratory of Assistant Investigator Shigeru Sakonju, Ph.D. (University of Utah). The gene structures for both *abd-A* and *Abd-B* have been characterized. The *abd-A* gene encodes a single protein that binds specifically to the promoter region of another homeotic gene, *Antennapedia*. The transcriptional regulation of *Antp* by *abd-A* in *Drosophila* is being investigated. Two genetic functions have been mapped to the *Abd-B* gene. Molecular analysis indicates that four overlapping transcripts are generated from the *Abd-B* gene. One transcript encodes a protein that provides a function needed in the fifth to eighth abdominal segments; the other three transcripts encode a protein required in the ninth abdominal segment.

Assistant Investigator Philip A. Beachy, Ph.D. (The Johns Hopkins University) and his colleagues have focused their studies on identifying morphogenetic genes that are involved in metameric pattern formation in *Drosophila*. Milligram quantities of the DNA-binding protein encoded by the homeotic gene *Ultrabithorax* (*Ubx*) have been purified, and methods have been developed to separate bound from unbound DNA molecules. Several *Drosophila* cell lines have been developed that express *Ubx* proteins under inducible control and provide an additional route to the identification of target genes and to testing the significance *in vivo* of the binding sites. Finally, several genomic regions have been found to contain potential targets for *Ubx* regulation through examination of the expression pattern of an introduced β-galactosidase gene.

Members of the laboratory of Investigator Michael W. Young, Ph.D. (The Rockefeller University) are studying the genes that control certain biological rhythms and the development of the nervous system. The distribution of the proteins expressed by two genes, *Notch* and *per*, has been determined. The Notch protein has been found on several cell types, chiefly of ectodermal origin, both before and after terminal differentiation. The distributions uncovered are consistent with their having a role in position-dependent development through the establishment and maintenance of specialized cell contacts among differentiating cells. Earlier genetic studies had demonstrated a requirement for per in the nervous system for the production of circadian rhythms. The per protein is produced in the eye and optic lobes of the brain and in the thoracic ganglia. The protein is also synthesized in the ring gland complex that produces a hormone that determines the timing of eclosion. Abundant expression is also seen in gonadal tissue.

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The molecular basis of metamorphosis in *Drosophila* is being studied by the laboratory of Assistant Investigator Carl S. Thummel, Ph.D. (University of Utah) by isolation and characterization of regulatory genes induced by the steroid hormone ecdysone. Current work is focused on one such gene, *E74*. The *E74* gene has a complex structure consisting of three overlapping transcripts. An ecdysone-responsive promoter directs the synthesis of a 60 kb primary transcript that is spliced to form the 6 kb *E74A* mRNA. The unusual length of this transcription unit delays the appearance of cytoplasmic *E74A* mRNA until one hour after the addition of ecdysone. This transcript contains an unusually long 5′-untranslated leader region, providing the potential for translocational regulation. Preliminary evidence suggests that this leader may function to delay further the expression of the *E74A* protein. Internal promoters direct the production of two *E74B* mRNAs. The *E74A* and *E74B* transcripts encode related proteins that have unique amino-terminal domains but share a common carboxyl-terminal domain. This common domain is highly basic and is 50% identical to the protein encoded by the *ets*-2 proto-oncogene. The protein encoded by the *E74A* mRNA binds to a single site in the middle of the *E74* gene. Biochemical studies to characterize the *E74* proteins are currently under way.

The human X and Y chromosomes have received considerable attention from geneticists. The Y chromosome appears to be critically involved in sex determination, and the X chromosome contains a number of genes that are important in various human genetic disorders, such as hemophilia, glucose-6-phosphate dehydrogenase deficiency, Duchenne muscular dystrophy, X-linked ichthyosis, and retinitis pigmentosa. In addition, an interesting type of regulation has been found to involve the X chromosome. Although females have two X chromosomes and males only one, the amounts of most X-encoded gene products are the same in both sexes. This is because one of the two X chromosomes in female cells is switched off very early in embryogenesis. The laboratory of Investigator Larry J. Shapiro, M.D. (University of California at Los Angeles) is interested in the mechanisms that produce this X chromosome inactivation as well as the processes responsible for the generation of mutations in X-linked diseases. Their research should help to clarify the structural organization of the sex chromosomes and their evolutionary history, as well as provide us with an understanding of the genetic basis of a number of human hereditary disorders.

Associate Investigator Yun-Fai Chris Lau, Ph.D. (University of California at San Francisco) and his colleagues have characterized two putative testis-determining genes, the zinc finger Y (*ZFY*) on the Y chromosome and the male-enhanced antigen (*MEA*) genes on chromosome 6. The cDNAs for the *ZFY* gene and a homologous gene (*ZFX*) on the X chromosome have been isolated and sequenced. Expression studies have demonstrated that the *ZFY* and *ZFX* genes are differentially transcribed as discrete-sized mRNAs in adult gonadal and somatic tissues, suggesting the possibility of separate functions for these genes. The expression of the mouse *Zfy* gene is linked to spermatogenesis in adult males. *Zfy* transcripts were also detected in fetal mouse testes during gonadogenesis. These observations support the hypothesis that the *ZFY* gene is analogous to the *TDF* gene in humans and that the *Zfy-1* gene is the *Tdy* gene of the mouse. In addition, testis-specific expression of the *ZFY* gene in adult males also suggests that it may play a role in regulating male germ cell development. Studies on transgenic mice that have the entire human *MEA* and linked genes indicate that the human genes are expressed at as high levels in adult testis as are the endogenous mouse genes and are subjected to the same tissue-specific regulation. Together with the previous finding of the expression of the mouse *Mea* and *Gene A* in fetal gonads, these data are consistent with the view that the *MEA/Mea* and related genes are important for mammalian spermatogenesis and/or testis differentiation.

A primary map of DNA markers for the entire human genome is almost complete. Once an unknown gene has been localized to a specific chromosomal region by family linkage studies with markers on the primary map, more closely linked markers can be developed for the region, to flank the locus in question and define the segment that needs to be searched for the gene. That level of characterization has been reached in the laboratory of Investigator Raymond L. White, Ph.D. (University of Utah) for two genes, those responsible for adenomatous polyposis coli and for von Recklinghausen (type 1) neurofibromatosis. Efforts to isolate and clone these genes and to characterize their functions are under way.

New mutations are a frequent cause of inherited disease. This is particularly true of some severe diseases and those that are due to mutations on the X

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chromosome. There are over 400 inherited diseases whose genes are known to be located on the X chromosome. Investigator C. Thomas Caskey, M.D. (Baylor College of Medicine) and his colleagues have developed a simple means of scanning these disease-producing genes for the mutation that is specific for a given family. These developments have made possible the accurate diagnosis of affected children as well as the identification of female carriers at risk of bearing affected males. Given this success, the laboratory is now embarking on a strategy for cloning the X chromosome and identifying more readily all disease-producing genes on the chromosome.

The laboratory of Associate Investigator Francis S. Collins, M.D., Ph.D. (University of Michigan) is also engaged in the search for genes responsible for human genetic diseases. Working with collaborators in Toronto, they have identified the cystic fibrosis gene and characterized the mutation that causes this disease, the most common genetic disorder of the Caucasian population. A similar set of strategies is being applied to identify the neurofibromatosis and Huntington disease genes. Efforts to understand gene regulation are also under way, both for the cystic fibrosis gene and for the human fetal hemoglobin genes.

Associate Investigator Louis M. Kunkel, Ph.D. (Children's Hospital, Boston) and his colleagues have increased their understanding of dystrophin, the protein product of the Duchenne/Becker muscular dystrophy locus. By injecting normal muscle cells into mutant mouse muscle, they have successfully replaced dystrophin in a mouse model of the disease. Dr. Kunkel's group is currently looking for patients with unexpected clinical symptoms for abnormalities of dystrophin. The work has now expanded into identification and isolation of proteins related to dystrophin in the anticipation that these proteins may be disrupted in other neuromuscular disorders.

The laboratory of Investigator Uta Francke, M.D. (Stanford University) has been engaged in assigning cloned genes of known functions for a variety of cell surface receptors, hormones, or regulatory proteins, as well as muscle-specific enzymes, to sites on human and mouse chromosomes. Panels of well-characterized somatic cell hybrids and *in situ* chromosomal hybridization techniques are being used. Recently, genetic mapping in the mouse has also been carried out with recombinant inbred strains for more precise localization on the genetic map. The comparative human and mouse maps are converging as more conserved syntenic regions are recognized. Based on gene localization, hypotheses have been formulated and are being tested regarding candidate genes involved in specific mutations or human inherited disorders. In addition, Dr. Francke's laboratory is attempting to determine the molecular basis for autosomal forms of muscular dystrophy that clinically resemble the X-linked classical Duchenne muscular dystrophy in families in which it has been shown that the dystrophin gene is not involved.

Phenylketonuria (PKU) is a genetic disorder of amino acid metabolism that causes severe and permanent mental retardation in untreated children. A number of PKU mutations associated with prevalent mutant phenylalanine hydroxylase alleles have been characterized in both Caucasians and Orientals by the laboratory of Investigator Savio L. C. Woo, Ph.D. (Baylor College of Medicine). At present, about 50% and 30% of PKU carriers, respectively, can be readily detected in the two populations. Once the 75% level has been reached by characterizing additional mutant alleles, screening can be implemented to reduce the carrier frequency by a factor of four. This reduction can theoretically lead to the reduction of PKU incidence from 400/year to only 25/year in this country.

Recombinant DNA techniques are being used by Investigator Arthur L. Beaudet, M.D. (Baylor College of Medicine) and his colleagues to study human genetic diseases. The exact mutations have been identified in numerous patients with citrullinemia, a fatal disorder of children caused by accumulation of ammonia in the body. Somatic gene therapy for citrullinemia also is being explored. The gene for spinocerebellar ataxia, a fatal human disease manifested by neurological deterioration and loss of muscle control in middle age, has been mapped by Dr. Beaudet's group to a small portion of human chromosome 6. The laboratory has also worked extensively to develop DNA tests for prenatal diagnosis, carrier detection, and population screening for cystic fibrosis.

Investigator David Valle, M.D. (The Johns Hopkins University) and his colleagues also focus on molecular studies of genes responsible for inborn errors of metabolism. In particular, his laboratory has examined the regulation, expression, and genetic defects of the gene encoding ornithine-δ-aminotransferase. Deficiency of this enzyme disrupts ornithine metabolism and causes an inherited form of blindness due to progressive chorioretinal de-

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generation, known as gyrate atrophy of the choroid and retina. The molecular basis and functional consequences of 15 mutations of this gene have been determined. The number and distribution of these mutations in a well-defined population group of Finns with a high frequency of gyrate atrophy have been determined, with the aim of understanding factors that influence the origin and spread of such mutations. Studies of the gene for phenylalanine hydroxylase have delineated a mutant allele that accounts for a substantial fraction of the mutations responsible for PKU in American blacks. Finally, with collaborators at The Johns Hopkins University, Dr. Valle has detected the first mutation in the $G_s\alpha$ gene, which causes pseudohypoparathyroidism, a dominantly inherited disorder in calcium homeostasis.

The laboratory of Assistant Investigator Cornelis Van Dop, M.D., Ph.D. (formerly Children's Hospital, Boston) has investigated the molecular basis of altered hormone responsiveness in human disease. The two primary diseases being studied are pseudohypoparathyroidism, which is manifested as resistance to parathyroid hormone, and idiopathic heart failure, a sporadic disease that has significant mortality and is the most common reason for heart transplantation. The clinical manifestations in both these diseases result in part from reduced responsiveness of the affected tissues to hormones and neurotransmitters that increase cAMP synthesis in the tissue. The studies of this laboratory have demonstrated reduced functional levels of specific membrane proteins that couple signals from hormone receptors on the outside of the cell across the cell membrane to the enzyme adenylyl cyclase that synthesizes cAMP inside the cell. The molecular mechanisms that affect this normal transmembrane signaling system in these diseases are being examined.

The genetics of two different human disorders have been studied by the laboratory of Investigator Jonathan G. Seidman, Ph.D. (Harvard Medical School). The chromosomal position of the locus responsible for familial hypertrophic cardiomyopathy (FHC) has been identified. This is an important first step toward identifying the gene responsible for this disorder and defining the molecular basis of cardiac hypertrophy. The role of somatic gene rearrangement of T cell receptor β-chain genes in an animal model of diabetes was examined. Studies of NOD (non-obese diabetic) mice bearing a rearranged functional T cell receptor β-chain transgene suggest that somatic gene rearrangement of β-chain genes is not required to cause this autoimmune disease.

An interest in the identification of genetic determinants of complex phenotypes in common disease has led the research group of Investigator Jean-Marc Lalouel, M.D., D.Sc. (University of Utah) to investigate the factors responsible for heritable disorders of lipid metabolism and early occurrence of myocardial infarction. At present work is focused on the molecular genetics of lipoprotein lipase, a key enzyme in triglyceride metabolism. Cloning and sequencing of the entire coding region of the gene has revealed two point mutations. The functional significance of the first mutation was established when lipoprotein lipase inactive, but immunoreactive, protein was found transiently *in vitro* in a system for gene expression. A relationship between carrier status and hypertriglyceridemia was demonstrated after molecular identification of carriers among relatives of the proband. Investigations will now focus on the possible role of lipoprotein lipase defects in familial hypertriglyceridemia and on the relationship between the structure and function of the enzyme.

The work of Assistant Investigator Stephen T. Reeders, M.D. (Yale University) involves approaches toward the elucidation, at the molecular or DNA level, of the defects associated with the development of autosomal dominant polycystic kidney disease and essential hypertension. The methodology employed, often termed the *reverse genetic approach*, allows investigators to work toward the isolation and study of the gene(s) responsible for these diseases, using as a starting point families in which one can demonstrate cosegregation of the disease with a previously defined genetic marker. Once cosegregation, or linkage, is observed, molecular biological approaches such as DNA cloning and sequence analysis from the region of the chromosome thought to be implicated are employed. Differences in the DNA from within this region between normal and affected individuals are then sought.

The laboratory of Investigator David M. Kurnit, M.D., Ph.D. (University of Michigan) employs a multifaceted approach to the study of Down syndrome and development. The use of molecular probes on chromosome 21 enables this group to examine the molecular structure of chromosome 21 and study the origins of trisomy 21 leading to Down syndrome, a leading cause of heart defects and mental retardation in humans. In addition, the study of expressed sequences on chromosome 21 enables the

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molecular pathology of Down syndrome to be elucidated. In other studies this laboratory has examined the determination of laterality, a fundamental event in embryogenesis. They have mapped a mutation that results in random determination of situs (position for the heart and other internal organs) to murine chromosome 21.

Methylmalonic acidemia (MMA) is an often fatal inborn error of metabolism that may arise from a deficiency of the enzyme methymalonyl CoA mutase (MCM). Cloning of the cDNA for MCM by the laboratory of Assistant Investigator Fred D. Ledley, M.D. (Baylor College of Medicine) has enabled characterization of the normal structure of the MCM enzyme and gene locus, classification of alleles underlying MCM deficiency, and identification of mutations in cells from patients with MMA. Mouse MCM has been cloned and characterized and is homologous to the human enzyme in structure and function. The present studies are directed toward elucidating the structure and function of MCM, the effect of mutations on enzyme activity, and the mechanisms by which MCM deficiency gives rise to a pathological phenotype.

An abnormality of chromosome 15 causes two different genetic diseases, Prader-Willi syndrome and Angelman syndrome. These genetic disorders have been found to be determined by the sex of the parent that transmits chromosome 15. The laboratory of the late Investigator Samuel A. Latt, M.D., Ph.D. (Children's Hospital, Boston) continues to investigate these disorders. The absence of the mother's chromosome 15 genetic material results in the Angelman syndrome, which is characterized by puppet-like movements and severe mental retardation, whereas absence of the father's chromosome 15 genetic material causes Prader-Willi syndrome, which is characterized by obesity and mild mental retardation. Further molecular studies of the abnormalities of the 15q11q13 subregion responsible for these syndromes and the phenomenon of genetic imprinting are under way.

Associate Investigator Robert L. Nussbaum, M.D. (University of Pennsylvania) and his colleagues study human heritable disease of unknown cause whose approximate genetic location is known from genetic mapping techniques. Examination of mental retardation with Xq27–28 fragile site (the fragile X syndrome) has demonstrated that the fragile site occurs at a region normally present in humans but one that undergoes an undefined alteration to produce the various mutations that can occur in families. Efforts are under way to isolate the region containing the fragile site in order to understand the molecular basis for the disorder. The laboratory also has localized the disease gene of the hereditary retinal disease choroideremia and has obtained DNA sequences very near the relevant gene that are now being examined for portions of the choroideremia gene. The laboratory has provided the first regional localization of the disease gene for Lowe syndrome by genetic linkage and has developed tightly linked, flanking markers for the disease locus. Work is in progress to isolate the gene responsible for the disease by isolating DNA from a chromosome translocation in which the breakpoint of the translocation marks the position of the Lowe syndrome gene.

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 and the basis of inherited disorders in which the function of these cells is altered. The mechanisms controlling gene expression in developing red blood cell precursors and the regulation and cellular biology of a system in white blood cells that produces bactericidal products are under study. In the past year the gene has been cloned for a major transcription regulatory protein for erythroid cells. This protein is believed to play a major role in controlling the expression of both globin and nonglobin genes in these cells. In addition, mutations have been defined in a novel white cell–specific cytochrome that lead to chronic granulomatous disease, an X-linked immune deficiency state in which microbes cannot be killed. The partial correction of this clinical disorder in some patients by the administration of a cytokine (interferon-γ) has led to a new approach to therapy.

Work in the laboratory of Assistant Investigator David A. Williams, M.D. (Children's Hospital, Boston) is directed at understanding blood cell formation and developing approaches to the treatment of human diseases affecting blood cells. Blood cells are derived from stem cells, primitive progenitor cells that reside in the bone marrow, and some human diseases are caused by the lack of specific proteins in these cells. This laboratory is studying the use of modified viruses to deliver functioning genes into bone marrow stem cells to provide these proteins and correct the deficiency.

The laboratory of Investigator Yuet Wai Kan, M.D. (University of California at San Francisco) continues to study genetic disorders of the red blood cell. Simpler and faster methods for prenatal diagnosis

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of sickle cell anemia are being developed so that the test can be performed in many countries where the disease is common. Mutations that cause thalassemia continue to be discovered, and the molecular basis of the control of globin gene expression is being investigated. The complex pattern of alternate splicing of the cytoskeleton protein 4.1 and the function of its different isoforms are also being delineated. A new form of post-transcriptional processing was described in the red cell enzyme glucose-6-phosphate dehydrogenase (G6PD). This protein is encoded by two genes on two different chromosomes. A single protein is made from these two genes post-transcriptionally, either by cross translation of the two mRNAs or by joining of the two polypeptides by transpeptidation.

Classic hemophilia is an inherited male-specific bleeding disorder resulting from a defect in a blood coagulation protein, factor VIII. The laboratory of Assistant Investigator Jane Gitschier, Ph.D. (University of California at San Francisco) has used the sensitive technique of denaturing gradient gel electrophoresis to find mutations in the factor VIII genes of hemophilia patients. Knowledge of these mutations can be helpful for understanding factor VIII function and in genetic counseling. The factor VIII gene is located at the tip of the long arm of the X chromosome and is closely linked to a number of genes associated with other inherited diseases, including a site responsible for a major cause of mental retardation in males. This year a large-scale map of the DNA in this region was constructed. The map should provide a basis for isolating other disease genes.

The research program of Assistant Investigator David Ginsburg, M.D. (University of Michigan) and his colleagues focuses on the biology of the human blood clotting system. Progress has been made in our understanding of the biology of von Willebrand factor (vWF) and the molecular basis of von Willebrand's disease (vWD), the most common inherited bleeding disorder in humans. Specific abnormalities were identified within the vWF gene that may cause one of the more common types of vWD and help to characterize how specific parts of the vWF molecule function in the blood clotting system. The group has continued also to study plasminogen activator inhibitor-1, a blood protein that plays a critical role in the body's system for breaking down blood clots. Abnormalities in this protein may contribute to a number of human diseases, including heart attack and stroke. Progress has been made also in the laboratory's study of bone marrow transplantation, an important treatment for leukemia and a number of other cancers.

Assistant Investigator Jeffrey M. Friedman, M.D., Ph.D. (The Rockefeller University) and his associates are studying the regulation and function of hormones and other molecules that are involved in the control of feeding behavior. They have taken two approaches to this problem. The first approach is directed at understanding, at the molecular level, the control of the mouse cholecystokinin gene. Cholecystokinin is a hormone that suppresses feeding behavior when administered to rats. This hormone, normally synthesized in the brain and intestine, has been found by this laboratory to be overexpressed in certain pediatric tumors, including Ewing's sarcoma of bone, neuroepithelioma (a chest wall tumor), and rhabdomyosarcoma (a muscle tumor). These observations may prove useful in the diagnosis and management of the tumors. The second approach of the laboratory is aimed at the molecular cloning of two mouse genes, *obese* and *diabetes*, which are defects in a single gene and result in profound obesity and abnormalities in feeding behavior. The proteins that code for these obesity genes have not yet been identified. The cloning of these genes and the characterization of the encoded proteins should provide insight into how food intake and body weight are regulated in mice and probably also in humans.

The laboratory of Associate Investigator Graeme I. Bell, Ph.D. (The University of Chicago) is using the techniques of genetics and molecular biology to identify and study the genes that contribute to the development of diabetes mellitus. Progress has been made in identifying the gene responsible for diabetes in a large family with maturity-onset diabetes of young people, a form of non-insulin-dependent diabetes mellitus. It is now possible to exclude this diabetogenic gene from about 12% of the human genome. The human insulin receptor gene is a very large gene, spanning over 120,000 base pairs. Its protein product mediates the cellular responses to insulin. The structural organization of the gene has been completed, and a general strategy has been developed that will facilitate the identification of mutations in this gene. Studies of the proteins responsible for the transport of glucose across the plasma membrane have revealed that there is a family of structurally related proteins that are responsible for glucose transport. Insulin regulates the amount of the unique glucose transport protein that is present in muscle and adipose tissue. As diabetes is characterized by an absolute or

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relative deficiency of insulin, it seems likely that decreased levels of this glucose transporter contribute to the increased blood glucose levels that are an important clinical feature of diabetes.

The laboratory of Associate Investigator Rebecca A. Taub, M.D. (University of Pennsylvania) is exploring three main areas of interest. 1) The genes and proteins that are expressed in liver cells immediately after the initiation of hepatic regeneration are being examined. Some of these genes may be responsible for the many subsequent steps occurring within a cell that result in cellular proliferation. 2) The laboratory is studying how the level of the insulin receptor gene expression is controlled within cells. In some diabetic conditions, decreased numbers of cellular insulin receptors may contribute to the diabetes. Knowing what controls the level of insulin receptors on a normal cell will facilitate understanding receptor abnormalities in diabetes. 3) The molecular makeup of antibodies directed against a receptor on platelets involved in blood clotting has been analyzed to learn about the region of the fibrinogen protein that normally binds to the platelet receptor.

Assistant Investigator Andrew P. Feinberg, M.D., M.P.H. (University of Michigan) and his colleagues are investigating the molecular genetics of human cancer and report progress in two areas of cancer genetics. The first involves alterations in DNA methylation, a modification of the nucleic acid cytosine in DNA that may play an important role in both normal gene regulation and the abnormal regulation that characterizes cancer. A novel system was developed for capturing cells in which the DNA is hypomethylated, but before the cells have become malignantly transformed. The laboratory has begun to identify genes that mediate the role of altered DNA methylation in carcinogenesis, as well as some of the earliest changes in gene expression in cancer. Second, the laboratory has localized tumor suppressor genes in several types of human cancer. Using molecular methods, they have identified several genetic alterations that may mediate the multiple steps in the development of colon and rectal cancer. They have also identified the location of the gene for Beckwith-Wiedemann syndrome, a disorder of abnormal growth that predisposes to several solid tumors of childhood. This gene is also involved in the progression of childhood and adult malignancies.

Research of Assistant Investigator Laimonis A. Laimins, Ph.D. (The University of Chicago) centers on the molecular biology of human papillomaviruses. Human papillomavirus (HPV) types 16 and 18 have been implicated as the causative agents of cancers of the cervix and penis. *In vitro* transformation studies have identified a new viral transforming gene E7 that together with the E6 gene product may play a principal role in the induction of cervical intraepithelial neoplasias by these viruses. *In vitro* alteration of differentiation is assayed in a system where epithelial cells are grown at an air-to-liquid interface, and papillomaviruses were observed to induce morphological changes similar to those seen in genital intraepithelial neoplasias *in vivo*. To examine the tissue tropism of HPV viruses, Dr. Laimins and his colleagues have studied the transcriptional regulatory mechanisms. Three distinct enhancer elements have been identified that may play different roles in the life cycle of these viruses. Furthermore, viral transactivators from herpes simplex virus, as well as a phorbol ester, have been shown to be capable of increasing papillomavirus expression. Studies are continuing on the structure and function of the viral proteins, as well as on a transgenic mouse model for HPV-induced malignancies.

Research in the laboratory of Assistant Investigator Donna L. George, Ph.D. (University of Pennsylvania) has led to the identification of one gene, and possibly another, with a potential for altering cellular growth patterns. In one case the introduction and subsequent overexpression of the gene in appropriate recipient cells results in a distinctive change in morphology characteristic of transformed cells. In the second case, a different expression pattern in transformed cells versus nontransformed cells may reflect a central role for this gene in some pathways of tumorigenesis. Progress also has been made in identifying DNA sequence elements important for the expression of the cKi-*ras* protooncogene and in isolating cDNA clones encoding their cognate DNA-binding proteins.

Growth factors induce a genetic program in target cells. Among the genes initially activated are several that encode known or probable transcription factors that are thought to regulate the genetic program, including Nur77, a new member of the steroid/thyroid hormone receptor family; Zif268, a zinc finger protein; and two members of the Jun family, c-Jun and Jun-B. Zif268 has been shown by Senior Investigator Daniel Nathans, M.D. (The Johns Hopkins University) and his associates to be a sequence-specific DNA-binding protein, and its consensus binding site has been defined. Jun-B, c-Jun, and a newly discovered member of the jun

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family (Jun-D) form homodimers (or heterodimers with each other) that bind to an AP-1 site or cAMP response element in DNA. However, Fos/Jun or Fra-1/Jun heterodimers have much greater affinity than Jun homodimers for an AP-1 site. In the Fos/Jun heterodimer the basic region of Fos contributes specific DNA-binding properties equivalent to those of Jun. These results support a model in which the Fos and Jun basic regions of the Fos/Jun heterodimer each interact with symmetrical DNA half-sites.

The laboratory of Assistant Investigator Vikas P. Sukhatme, M.D., Ph.D. (The University of Chicago) has identified novel genes whose expression is controlled by growth factors. One such gene, Egr-1, predicts a protein structure characteristic of a transcriptional regulator, i.e., a molecule capable of regulating the expression of a target gene(s). Thus these molecules can act as sensors of changes in the environment of a cell and serve to transmit and convert that information into long-term adaptive changes, such as decisions to divide or to differentiate.

The work of Associate Investigator Stephen A. Liebhaber, M.D. (University of Pennsylvania) and his colleagues focuses on the signals that determine the accuracy of the splicing of the initial RNA transcript into a functional mRNA, and the signals, both primary sequence and folding (secondary structure), that determine how efficiently the mRNA is translated into its final protein product. In addition, this laboratory is characterizing a newly discovered growth hormone gene that is specifically expressed by the placenta. This hormone has activities that suggest it may play an important role in fetal growth and/or maternal adaptations.

The main research interest of Investigator Robert Tjian, Ph.D. (University of California at Berkeley) and his associates concerns the means by which genetic information stored in DNA is retrieved in a controlled and orderly fashion during the biochemical process of transcription. They have taken a biochemical approach to the problem of gene control and have devised various means of isolating the individual components of the cell responsible for transcription. By reconstructing this complex reaction in the test tube they have been able to study how specific genes are turned on and off during cell growth and development—mechanisms that are of fundamental importance in understanding the normal metabolic processes that maintain living cells. Dr. Tjian's laboratory has defined novel protein structural domains responsible for specificity of DNA binding, protein dimerization, and transcriptional activation; they are exploring promoter-selective gene activation in animal cells; and they are defining new regulatory factors in the embryonic development of *Drosophila* and especially its nervous system.

The determination of cellular phenotype is a consequence of the control of gene expression in a particular cell under a particular set of circumstances. Thus an understanding of the mechanisms regulating gene expression, at whatever level it occurs, is crucial to a final understanding of these complex cellular changes. To this end, systems that permit the study of transcriptional regulation as well as post-transcriptional control of RNA processing have been utilized by the laboratory of Investigator Joseph R. Nevins, Ph.D. (Duke University). Cellular transcription factors that are the targets of viral regulatory proteins and are involved in the regulation of transcription of viral and cellular genes have been identified and purified. Cell-free systems have been established for studying this control *in vitro*, so as to better understand the biochemical mechanisms involved. A similar approach has identified factors involved in the processing of a primary transcript to yield a poly(A) site, another regulated event of gene expression in animal cells. These studies should provide a framework for exploring the signaling pathways within cells that ultimately regulate gene expression.

Assistant Investigator Harinder Singh, Ph.D. (The University of Chicago) had previously developed a new strategy for isolating genes that encode proteins that regulate the activities of other genes. With this strategy the human gene for a protein (Oct-2) that controls the activity of antibody genes in B cells was isolated. This laboratory has initiated studies on the structure, function, and regulation of Oct-2 in antibody-producing cells of the mouse, since this system can be manipulated experimentally.

Assistant Investigator Gary J. Nabel, M.D., Ph.D. (University of Michigan) and his laboratory are investigating the molecular regulation of viral and cellular genes, with emphasis on the expression of human retroviruses in T lymphocytes. Cellular factors that control transcription of these genes have been defined, including NF-κB, which activates the enchancer of the human immunodeficiency virus (HIV). Studies of HIV activation and NF-κB in the monocyte lineage show that NF-κB is activated at a discrete stage of monocyte differentiation and is as-

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sociated with HIV replication. The mechanism of NF-κB–mediated activation and the role of the TATA box have been defined, and a cDNA clone that encodes a κB-like–binding protein has been identified.

Characterization of the immunoglobulin heavy-chain enhancer has continued in the laboratory of Assistant Investigator Thomas R. Kadesch, Ph.D. (University of Pennsylvania). Two protein-binding sites have been identified that, together, recapitulate a negative mode, cell-type-specific regulation. One site binds a constitutively active transcription factor whose activity is specifically inhibited in non–B cells in the presence of the second site. Thus far this laboratory has isolated one cDNA encoding a protein that binds the first site and three distinct cDNAs (encoded by different genes) encoding proteins that bind the second site. All of the encoded proteins function as positive-acting transcription factors and possess structural motifs that facilitate protein-protein interactions.

Histocompatibility molecules are responsible for the binding and presentation of viral, bacterial, and tumor antigens to the immune system and for transplant rejection. In order to bind a large number of these antigens and ensure the survival of the species, it is beneficial that many varieties of the histocompatibility molecules be found in the population. The microrecombination process generates variety by reassorting genetic information among histocompatibility genes and other related genes in germ cells. This has been studied previously by isolation of mutant mice that differed from their parents in histocompatibility genes. The laboratory of Assistant Investigator Jan Geliebter, Ph.D. (The Rockefeller University) is investigating the microrecombination process by analyzing germ cells of normal mice. Since thousands of eggs or millions of sperm can be obtained from a single mouse, the group can analyze the equivalent of millions of mice for microrecombinations. These studies should enhance our understanding of the genetic processes that control the evolution and ultimately the function of the mammalian immune system.

The mechanism of tolerance to peripheral antigens has been examined by the laboratory of Investigator Richard A. Flavell, Ph.D. (Yale University). In these studies transgenic mice were used in which expression of the class II major histocompatability complex (MHC) molecule I-E was directed to the β islet cells of the pancreas (using the insulin promoter) and to the acinar tissue of the pancreas (using the elastase promoter). Mice carrying this peripherally expressed MHC are tolerant to the antigen, but the tolerance does not appear to result from clonal deletion of T cells that may be reserved for tolerance to MHC molecules that are expressed in the thymus. Instead, at least in the case of the insulin transgenic mice, these initial data suggest that tolerance results from a clonal paralysis of I-E–reactive T cells. In contrast to those cells from normal mice, I-E–reactive cells from the transgenic mice appear to be incapable of activation by exposure to antigen. These results suggest that the mechanism of tolerance to peripheral antigens may, at least in part, result from nondeletion mechanisms.

Assistant Investigator Stephen V. Desiderio, M.D., Ph.D. (The Johns Hopkins University) and his colleagues continue their work on the development of the immune system. One area of study is the assembly of antibody genes. This recombination reaction has been examined in detail, and a novel pathway for antibody gene rearrangement has been identified that has important implications for the mechanism of rearrangement and may serve to increase the diversity of the immune response. A conserved DNA sequence element, which accompanies all antibody genes, is required for efficient rearrangement and is the recognition site for a specific DNA-binding protein that has been purified to homogeneity and may represent a component of the recombination machinery. The laboratory also is examining transduction of growth and differentiation signals in cells of the immune system. They have found and characterized a novel gene that encodes a specific type of signal-transducing protein, similar in structure to a number of known oncogenes. The structure and restricted pattern of expression of this gene suggest that its product transduces a signal for growth and/or differentiation of antibody-producing cells.

The primary research focus of the laboratory of Assistant Investigator John B. Lowe, M.D. (University of Michigan) has been to investigate mammalian genes that determine the expression of cell surface carbohydrate molecules. These efforts have exploited experimental systems developed in Dr. Lowe's laboratory to isolate these genes without first purifying the proteins they encode. Three distinct glycosyltransferase genes have been isolated. These include the human genes that determine expression of the H and Lewis blood groups. Studies designed to answer questions about the functions of these genes during the early development of mammalian organisms are under way.

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The work carried out by Investigator Bernardo Nadal-Ginard, M.D., Ph.D. (Children's Hospital, Boston) and his colleagues is oriented toward the elucidation of the genetic mechanisms responsible for the production of muscle cells. The main component of these cells is the contractile apparatus that confers the functional properties of skeletal and cardiac muscle. The genes that encode for the contractile proteins are being analyzed by the laboratory. Once these genes are activated, they produce several different versions of the relevant contractile proteins, each of which is functionally different and may change in response to different physiological and pathological demands. The generation of different contractile systems is possible, because several genes produce different versions of the same protein and, in many cases, the same gene can produce a large number of variants by the process of alternative mRNA splicing. The molecular mechanisms responsible for these two modes of regulation are under investigation.

Assistant Investigator Jeremy Nathans, M.D., Ph.D. (The Johns Hopkins University) and his coworkers are studying the molecular mechanisms underlying our sense of sight and the genetic alterations that cause inherited variations in that sense. They have concentrated on the visual pigments, the light-sensitive proteins in the eye that mediate vision, and have recently determined the nature of the alterations that cause blue cone monochromacy, a rare defect characterized by a complete absence of color sense, low acuity, nystagmus (involuntary eye movements), photophobia, and in some cases a progressive scarring of the central retina. In most affected individuals, sequences adjacent to the genes encoding the red- and green-sensitive pigments are deleted. Most likely, the deleted region contains DNA sequences that control expression of these genes in the retina.

The human immunodeficiency virus type 1 (HIV-1) encodes a regulatory protein, Rev, that is essential for viral replication. The laboratory of Assistant Investigator Bryan R. Cullen, Ph.D. (Duke University) has shown that the role of Rev is to activate viral structural protein expression by facilitating the nuclear export of viral mRNA molecules within the infected cell. Mutational analysis of the *rev* gene suggests the existence of two functional protein domains—one that binds directly to a viral mRNA target sequence and a second that interacts with the cellular RNA transport machinery. Defective Rev proteins that lack this second domain but continue to bind viral mRNAs have now been shown to inhibit Rev function and hence to prevent HIV-1 replication. These trans-dominant repressors of Rev function may prove useful in strategies for gene therapy as an approach to the treatment of AIDS (acquired immune deficiency syndrome).

The laboratory of Assistant Investigator Patrick O. Brown, M.D., Ph.D. (Stanford University) is examining the mechanism by which a retrovirus can integrate its genes into a chromosome of its host cell. Integration is an essential step in retroviral reproduction and may provide a tool for the therapeutic introduction of genes into human cells. Dr. Brown and his colleagues have determined the structure of two key intermediates in integration and have identified a specific biochemical step carried out by a viral protein. Extending their previous studies on a murine retrovirus, this group has developed a test-tube method for studying the integration of HIV.

The laboratory of Assistant Investigator John W. Belmont, M.D., Ph.D. (Baylor College of Medicine) has focused on the investigation of retroviral gene transfer into hematopoietic stem cells. The primary application of these studies is in the development of a clinically useful method for gene transfer therapy. Retroviral vectors are being utilized because of their potential for efficient gene transfer. These studies are also aimed at defining some of the biological properties of hematopoietic stem cells and their lymphoid progeny.

Chemical reactions necessary for life are typically catalyzed by enzymes. These large molecules are usually proteins, but recently it has been found that RNA, which was previously thought to be only an information-carrying molecule, can act as an enzyme. In the past year a more detailed view of the mechanism of one example of RNA catalysis has been obtained by Investigator Thomas R. Cech, Ph.D. (University of Colorado at Boulder) and his colleagues. For example, the release of the product from the active site was found to limit the speed of the reaction, and the reverse direction of the reaction was observed for the first time. A new method was also developed to determine which parts of the RNA molecule are on its surface and which are buried in the interior. The result is a clearer picture of the overall molecular structure.

The laboratory of Associate Investigator Jeffry L. Corden, Ph.D. (The Johns Hopkins University) has been studying RNA polymerase, the enzyme that synthesizes mRNA copies of activated genes. RNA polymerase contains an unusual repeated amino acid sequence that is modified by the enzy-

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matic attachment of phosphate. An enzyme that phosphorylates RNA polymerase has been purified. One of the components of this enzyme is a protein that has previously been implicated in controlling the cell division cycle. This result may lead to a greater understanding of the genetic control of cell growth and the role RNA polymerase plays in this process.

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 Lindquist, Ph.D. (The University of Chicago) and her colleagues focuses on three aspects of the response. First, the rapid and reproducible induction of new proteins is under study as a general model system to investigate mechanisms of genetic regulation in higher organisms. The group has shown that the major heat-shock protein, hsp70, is repressed during recovery from heat shock by a mechanism that recognizes the 3′ end of the hsp70 messenger RNA and targets it for degradation. Second, the laboratory is examining the mechanisms that cells employ to protect themselves from heat stress. They have found that one of the heat-shock proteins, hsp83, is essential for all temperatures but is especially required at higher concentrations for cell growth at higher temperatures. They have also found that a third protein, hsp106, is required for cells to tolerate short-term exposure to extreme temperatures without dying. Finally, the laboratory is interested in some practical applications of the heat-shock response. They have used the heat-shock promoter and translation signals to express the site-specific recombination system of the yeast 2μ plasmid in *Drosophila*. The induction of the recombinase by heat shock induces site-specific recombination at recombination target sequences embedded in the *Drosophila* genome. This system should enhance methods of genetic analysis in *Drosophila* and is also likely to be applicable to other organisms.

The laboratory of Investigator Joan Argetsinger Steitz, Ph.D. (Yale University) is continuing to investigate how a number of recently discovered small particles contribute to basic cellular processes. These particles contain RNA and protein and play essential roles in the multiple steps by which 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 such rheumatic diseases as systemic lupus erythematosus. Understanding the nature of the particles is therefore important not only for basic molecular biology but also for improving the diagnosis and treatment of rheumatic disease.

The diversity of unusual translation events that ribosomes display continues to occupy a major portion of the efforts of the laboratory of Investigator Raymond F. Gesteland, Ph.D. (University of Utah). Most retroviruses couple the synthesis of the GAG protein to the *gag-pol* precursor for the polymerase through the occasional slipping by ribosomes into one of the alternative reading frames at special sequences at the *gag-pol* junction. Fortunately these special sequences also cause bacterial ribosomes to make the same change in the reading frame following the same rules, and this provides an opportunity to work out the details of the reaction and to screen drugs efficiently for those that alter ribosomal frameshifting. Rules for ribosome jumping are more complicated. The large jump that ribosomes use to navigate past 50 nucleotides of untranslated mRNA during expression of the bacteriophage T4 gene *60* requires a long stretch of upstream information. Surprisingly this turns out to be the peptide sequence of the growing chain that somehow interacts with the ribosome to tell it to jump. Study of these unusual events continues to reveal more secrets of the complicated ribosomal machinery of the cell.

The major project under way in the laboratory of Assistant Investigator George M. Church, Ph.D. (Harvard Medical School) is directed at facilitating the sequencing of small genomes to compare them for conserved elements. To accomplish this rapidly and accurately, new sequencing tools have been developed. One of these, multiplex sequencing, is a way of keeping a large set of DNA fragments as a precise mixture throughout most of the steps of sequencing. Machines and software have been developed that integrate automatic base assignment routines with high-resolution image display and interactive multisequence alignments. Images with overlapping data can be easily retrieved to facilitate building and checking the final consensus sequences. The technology is being applied in Dr. Church's laboratory to the sequencing of two bacterial genomes, *Escherichia coli* and *Salmonella typhimurium*.

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The genes for several bacterial histone-like proteins have been identified in *Salmonella typhimurium* and are under study in the laboratory of Assistant Investigator David R. Hillyard, M.D. (University of Utah). Mutations in *hup*A and *hup*B confer defects in bacteriophage Mu transposition, flagellar phase variation, and F plasmid stability. Mutant strains that make none of the major histone-like protein HU, show alterations in plasmid supercoiling and compensatory increases in other nucleoid proteins. Work has begun on a novel conotoxin from venomous sea snails that binds specifically to glutamate receptors of the NMDA variety in the central nervous system of vertebrates.

CONTROL OF MORPHOGENESIS BY THE *DROSOPHILA* HOMEOTIC GENE *ULTRABITHORAX*

PHILIP A. BEACHY, PH.D., *Assistant Investigator*

The body plan of *Drosophila*, like that of many metazoa, is characterized by a series of homologous segments, or metameres, that bear structures specialized for feeding, locomotion, reproduction, and other functions. This anatomical format results from the sequential deployment during embryonic development of several hierarchically arranged groups of genes. The products of many of the genes within this hierarchy have been implicated in transcriptional control, either through direct biochemical studies or by the presence of certain structural motifs associated with DNA-binding activity. Little is known, however, of the mechanisms by which regulatory instructions issued from within the nucleus control morphogenetic pathways leading to the diverse morphologies of individual segments. Dr. Beachy's laboratory is attempting to identify morphogenetic genes in *Drosophila* that are targets for the products of genes within regulatory levels of the hierarchy and to understand the mechanisms that govern expression of these target genes.

Research is focused on the *Drosophila* homeotic gene *Ubx* (*Ultrabithorax*), which, together with half a dozen other homeotic genes, occupies the lowest and latest-acting tier of the pattern formation hierarchy. The homeotic genes act within a repetitive segmented framework established by genes within higher tiers of the hierarchy to produce the specialized structures that distinguish the segments. *Ubx* is primarily responsible for the distinguishing features of a contiguous region including parts of two thoracic segments and one abdominal segment. Past work has demonstrated that *Ubx* encodes a family of closely related nuclear proteins with sequence-specific DNA-binding properties; these proteins are capable of modulating transcription *in vivo*, and transcriptional control is likely to be the primary mechanism of *Ubx* and other homeotic gene action during development. With this as a starting point, Dr. Beachy's laboratory is pursuing several strategies for the identification of target genes.

I. Isolation of Tightly Bound DNA Sequences.

The effects of *Ubx* regulation are likely mediated through the interactions of *Ubx* proteins with specific DNA sequences located near target genes. One approach to the isolation of target genes therefore would utilize *Ubx* protein as a reagent to identify and isolate genomic DNA sequences bound with high affinity. A large-scale procedure based on specific-sequence DNA affinity chromatography has been developed that yields milligram quantities of nearly homogeneous *Ubx* protein from an overproducing *Escherichia coli* strain. Conditions for nitrocellulose filter-binding and gel-retention methods have been developed that should permit the isolation of specific protein complexes with *Drosophila* genomic DNA fragments.

II. Isolation of Genes and Promoters Responsive to *Ubx* Expression.

This laboratory has developed several clonally derived cell lines in which *Ubx* expression is under control of the *Drosophila* metallothionein promoter. Conditions have been established in which *Ubx* proteins are induced and maintained at intracellular levels similar to those in embryos. It should now be possible to isolate sequences differentially expressed in the induced and uninduced state; these sequences will be tested for proximity to *Ubx* protein-binding sites, perhaps by cross-hybridization to isolated genomic sequences.

To identify promoters that respond to *Ubx* expression in embryos, several hundred strains, each containing an independent insertion of a P element–based "enhancer sniffer," have been examined by histochemical staining of embryos for β-galactosidase activity. The enhancer sniffers contain the *E. coli* β-galactosidase gene under control of a weak promoter, whose activity is strongly influenced by genomic context. Many of the strains display tissue- or cell-specific staining patterns; of particular interest is a subset of these that show cell-specific patterns with clear segmental differences, because these insertions may identify regulatory sequences responsive to homeotic genes. These strains will be examined for altered expression patterns in genetic backgrounds where *Ubx* protein expression is altered. For strains that merit further study, *Drosophila* genomic DNA flanking the site of insertion can be isolated by marker rescue, using an antibiotic resistance gene and a plasmid origin within the enhancer sniffer.

III. DNA Looping and the Mechanism of *Ubx* Action.

Protein-mediated DNA looping has been suggested as a mechanism by which eukaryotic enhancers and certain regulatory sequences in pro-

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karyotes exert their effects over large distances. This laboratory has obtained electron microscopic and biochemical evidence for *in vitro* formation of a DNA loop mediated by *Ubx* protein binding to two sites near the *Ubx* transcription start site. Formation of this loop may be aided by the presence of intrinsically curved sequences in the spacer DNA between the two sites. DNA constructs in which various combinations of the two binding sites and the spacer have been mutated or rearranged are being tested *in vitro* for effects on loop formation. Although there is some evidence for autoregulation, the potential role of this loop *in vivo* is more difficult to assess, since normal *Ubx* expression in embryos requires extensive upstream sequences; this creates difficulty in handling. The behavior of these mutations is therefore being assessed in cultured cells, using a smaller and more manageable *Ubx* promoter fragment. The laboratory is particularly interested in the possibility that loop-based mechanisms may be involved in the regulation of downstream target genes.

Dr. Beachy is also Assistant Professor of Molecular Biology and Genetics at The Johns Hopkins University School of Medicine.

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MOLECULAR STUDIES OF HUMAN GENETIC DISEASES

ARTHUR L. BEAUDET, M.D., *Investigator*

Research in Dr. Beaudet's laboratory is focused on molecular approaches to a number of human genetic diseases, including spinocerebellar ataxia, cystic fibrosis, deficiencies in urea cycle enzymes, and disorders of cell adhesion molecules. Much of this research is conducted in collaboration with Dr. William E. O'Brien (HHMI Senior Associate).

I. Argininosuccinate Synthetase Locus and Citrullinemia.

Genetic deficiency of argininosuccinate synthetase (AS) causes neonatal citrullinemia, which is characterized by increased blood ammonia, mental retardation, and early death without treatment. Seven single-base missense mutations causing citrullinemia were identified by sequencing clones obtained by amplification of cDNA from cultured fibroblasts. Four additional alleles are associated with the absence of a single exon sequence in the cDNA, and another mutation is a single-base splicing abnormality resulting in the absence of seven base pairs in the cDNA sequence. The characterization of these 12 mutant alleles indicates that the mutations causing citrullinemia are extremely heterogeneous, and all nonconsanguineous patients studied to date are compound heterozygotes. The mutation causing citrullinemia in Friesian cattle was identified as a nonsense mutation, at codon 86, and a heterozygote detection method using the polymerase chain reaction for analysis of genomic DNA was developed.

DNA polymorphisms were identified within the human AS gene and were used to map the gene more precisely through the CEPH (Centre d'Etude du Polymorphisme Humain) collaborative linkage study. The human gene maps to chromosome 9q34 close to the ABO blood group with a recombination fraction of 0.04. These polymorphisms also provide improved methods for prenatal diagnosis of citrullinemia. Similar mapping studies were performed in the mouse, using recombinant inbred strains. The mouse gene maps to chromosome 2 with close linkage to the fifth component of complement, placing the gene in a conserved linkage group between mouse and human, including the fifth component of complement, the AS gene, the Abelson oncogene, and adenylate kinase-1.

Studies aimed at developing somatic gene therapy for various human diseases are being performed, using the AS gene and citrullinemia as a model system. Although this gene is usually expressed at high levels in hepatocytes, it is possible that expression in bone marrow–derived cells would provide an adequate correction. The human cDNA sequence was introduced into a modified N2 vector for expression from the long terminal repeat (LTR) with the viral structure LTR-cDNA-LTR. Retroviral titers of $3–5 \times 10^6$ were obtained using the GP+E-86 (ecotropic) and GP+*env*AM12 (amphotropic) packaging cell lines. Both packaging cell lines have been used for infection of mouse bone marrow cells, followed by reimplantation of bone marrow into lethally irradiated recipient mice. All mice express human AS activity in mouse peripheral blood. Most mice experience decreased levels of expression 8–15 wk post-transplantation, but many mice continue to express at significant levels beyond 20 wk. Retroviral infection of human and baboon bone marrow cells is being studied *in vitro* in preparation for *in vivo* experiments in baboons. Work is under way with a goal of developing a mouse mutant for citrullinemia. The mouse gene has been extensively characterized, and recombinant DNA constructions suitable for homologous recombination in embryonic stem (ES) cells are being prepared.

II. Gene Cloning for Spinocerebellar Ataxia.

Spinocerebellar ataxia (SCA 1) is a dominantly inherited neurodegenerative disorder that is mapped to the short arm of chromosome 6. Dr. Huda Zoghbi has performed extensive studies to map regionally the SCA 1 locus on chromosome 6p. A detailed somatic cell hybrid panel for regional mapping on chromosome 6p was developed. A genomic DNA library was prepared from a somatic cell hybrid containing chromosome 6p as the only identifiable human chromosomal material. Over 100 clones from this library were isolated and regionally mapped. Subsequently a set of radiation-induced hybrid cells was isolated containing small portions of chromosome 6p. Current data indicate that the SCA 1 locus maps centromeric to the HLA region and that flanking DNA markers are available. Efforts continue to map the SCA 1 locus precisely, with the goal of cloning the gene.

III. Molecular Genetic Studies of Cystic Fibrosis.

Cystic fibrosis (CF) is the most frequent lethal autosomal recessive disease in Caucasians, and the

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gene is mapped to chromosome 7q. Extensive studies of a North American population demonstrated strong linkage disequilibrium with the markers KM-19 and XV-2c, such that individuals of the highest risk genotype are 80 times more likely to carry the CF mutation than individuals of the lowest risk genotype. The laboratory has described detailed methods for use of linkage disequilibrium for diagnosis of genetic diseases, using CF as a model. The laboratory extended the application of the polymerase chain reaction for rapid molecular analysis of samples for prenatal diagnosis and carrier detection by establishing this method for polymorphisms detected by the KM-19, XV-2c, and J3.11 probes. More recently the laboratory has analyzed hundreds of samples using direct detection of the most common mutation causing CF, in collaboration with Drs. Francis S. Collins (HHMI, University of Michigan Medical School) and Lap-Chee Tsui (Toronto), who cloned the gene for CF. Direct analysis for the most common mutation identifies the defect in 70–75% of CF chromosomes, represents a major advance for prenatal diagnosis and carrier detection, and makes it possible to begin population-based carrier screening for CF.

IV. Molecular Studies of Cell Adhesion Molecules.

Numerous adhesion molecules on the surface of leukocytes and endothelial cells are being identified as the result of work in many different laboratories. The leukocyte-integrin complex is a major cell adherence molecule on the surface of granulocytes and other leukocytes, and genetic defects in the β-subunit of this integrin cause human leukocyte adhesion deficiency, a fatal granulocyte disorder. The mutation in one patient with leukocyte adhesion deficiency was identified as an ATG to AAG change in the initiation codon. A major goal of work in the laboratory is to develop mouse mutants for numerous leukocyte and endothelial cell adhesion molecules using homologous recombination in ES cells. Toward this end, the cDNA for the β-subunit of the murine leukocyte integrin and the cDNA for the murine intercellular adhesion molecule-1 (ICAM-1) were cloned and sequenced. Murine genomic clones for both genes were isolated, and recombinant DNA constructs suitable for homologous recombination in ES cells are being prepared. It is expected that genetic variation in the leukocyte and endothelial cell adhesion molecules will be of major importance in the pathogenesis of inflammatory disorders and vascular disorders such as vasculitis and atherosclerosis.

Dr. Beaudet is also Professor in the Institute for Molecular Genetics and the Departments of Pediatrics and of Cell Biology at Baylor College of Medicine.

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MOLECULAR BIOLOGY OF DIABETES MELLITUS

GRAEME I. BELL, PH.D., *Associate Investigator*

Dr. Bell's laboratory is studying the molecular biology and genetics of human endocrine disorders. Its primary interest is non-insulin-dependent diabetes mellitus (NIDDM).

I. Molecular Biology and Genetics of Non-Insulin-Dependent Diabetes Mellitus.

About 10% of adults in the United States are affected by NIDDM, a disorder of carbohydrate metabolism. NIDDM is characterized by fasting hyperglycemia, which if untreated contributes to the development of the chronic complications of diabetes mellitus and results in early mortality. Both genetic and nongenetic factors contribute to the development of this disorder. In most families the mode of inheritance of NIDDM does not conform to that of either a dominant or recessive disorder, thereby confounding genetic studies. NIDDM is also likely to be heterogeneous genetically, with a number of different diabetogenic genes segregating in human populations; any one of these genes may increase or even decrease susceptibility to this disorder. The goal of this laboratory is to identify these diabetogenic genes. A strategy that includes molecular biology and genetics is being used to help unravel the etiology of this disorder.

Dr. Stefan S. Fajans (University of Michigan) has described several families with a slowly progressing form of diabetes that occurs in some children, adolescents, and young adults and has a strong familial association. This form of NIDDM, maturity-onset diabetes of young people (MODY), is characterized by an autosomal-dominant mode of inheritance and is a good model for investigating the natural history of NIDDM. The largest and most thoroughly studied of such families is the RW pedigree; this family offers a unique opportunity to use reverse genetics to identify a diabetogenic gene. The MODY form of NIDDM in this family is characterized by insulinopenia and impaired β-cell function. Forty-five restriction fragment length polymorphisms (RFLPs) associated with 32 different loci have been tested for linkage to the MODY phenotype. A molecular marker for this disorder has not been identified. Two-point analyses exclude ~6% of the human genome from consideration. Since MODY is an autosomal-dominant disorder, the X and Y chromosomes can also be excluded from consideration; thus the MODY gene can be excluded from 12% of the human genome. Linkage studies in this family are continuing.

Genetic variation in the human insulin receptor gene is associated with insulin resistance and diabetes. Six missense mutations, one nonsense mutation, and one deletion have been described in the insulin receptor gene. These mutations provide insight into the structure and function of this protein. For example, identification and characterization of a mutation causing an Arg→Ser substitution at residue 735 in the proreceptor-processing site indicated that proteolytic processing of the proreceptor is necessary for its normal full insulin-binding sensitivity and signal-transducing activity. The human insulin receptor gene and its promoter have been isolated and characterized. The gene spans >120,000 base pairs (bp) and has 22 exons varying in size from 36 bp to >2,500 bp. The sequence of ~13,000 bp of the human insulin receptor gene has now been determined; this represents ~10% of the total gene. Using the sequences of the introns and flanking regions as a guide, Dr. Bell has selected primer pairs specific for each of the 22 exons and used the polymerase chain reaction (PCR) to amplify each exon specifically. The PCR and direct sequencing of the amplified DNA are being used to examine the sequences of both alleles of the insulin receptor gene of two patients who are extremely insulin resistant: one patient has the type A syndrome of severe insulin resistance, and the other has lipoatrophic diabetes.

The isolation and characterization of cDNAs/genes encoding proteins that may contribute directly or secondarily to the development of glucose intolerance and its metabolic sequelae have continued, in the belief that these genes may represent primary susceptibility determinants or that their altered regulation in the diabetic state contributes to the metabolic derangements characteristic of this disorder. These clones also represent molecular probes that can be used for genetic studies as well as for physiological studies examining the effects of diabetes on cellular processes.

The isolation and characterization of cDNAs encoding human facilitative glucose transporters have revealed that facilitative glucose transport is not the property of a single protein but is a feature of a family of structurally related proteins that have distinct but overlapping tissue distributions. These proteins have been designated glucose transporter

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(GLUT; this abbreviation is also the gene symbol) 1 to 5. Altered expression of the GLUT4 protein appears to contribute to the insulin resistance characteristic of insulin-deficient states such as diabetes mellitus and fasting.

Dr. Bell and his co-workers have also isolated and characterized cDNAs encoding other candidate diabetogenic genes. These include cDNAs encoding three different isoforms of human hexokinase and one isoform of human glucokinase; phosphorylation of glucose by these enzymes represents a key regulatory step in its intracellular metabolism. In addition, in collaboration with Dr. Donald F. Steiner (HHMI, The University of Chicago), cDNAs encoding islet amyloid polypeptide (IAPP) from human, rat, mouse, cat, and guinea pig have been isolated. IAPP is a component of the amyloid deposits present in the islets of Langerhans of diabetic patients. These deposits are believed to impair the function of the insulin-producing β-cell and thereby contribute to development of glucose intolerance. The contribution of each of these candidate genes to the natural history of NIDDM is being examined.

II. Other Projects.

In collaboration with Drs. Samuel Refetoff and Leslie DeGroot (The University of Chicago), the molecular basis for generalized resistance to thyroid hormone in one family has been determined. A G→C nucleotide replacement resulting in a Gly→Arg substitution at amino acid 340 in one of the two alleles of the patient's thyroid hormone β-gene results in the expression of a protein that does not bind thyroid hormone *in vitro*. This mutation is associated with delayed verbal expression and attention-deficit hyperactivity disorder.

Dr. Bell is also Associate Professor in the Departments of Biochemistry and Molecular Biology and of Medicine at The University of Chicago.

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TECHNIQUES FOR GENE THERAPY

JOHN W. BELMONT, M.D., PH.D., *Assistant Investigator*

I. Transfer of Human Adenosine Deaminase into Murine Hematopoietic Cells.

Dr. Belmont's laboratory has chosen the human adenosine deaminase (*ADA*) gene as a convenient biochemical marker for gene transfer into murine bone marrow cells. One form of human severe combined immune deficiency is caused by ADA deficiency. This is a rare autosomal recessive disorder, but it appears to be a good model for gene therapy research, because the molecular pathophysiology is relatively well characterized, and the disease is sufficiently severe to warrant investigation of experimental therapies. Extensive characterization in the murine model system should allow a smooth transition of promising gene transfer vectors into large animal and human experiments.

A promising basic vector had previously been identified that efficiently transduced human ADA into mouse hematopoietic progenitors. That vector utilizes the Moloney murine leukemia virus long terminal repeat (LTR) sequences to provide the promoter and enhancer functions for expression. Initial experiments indicated that this vector could be used to infect pluripotent hematopoietic stem cells and allow expression of the human ADA enzyme in their differentiated progeny of all lineages. This vector has been reevaluated in transplant experiments using virus produced from a new packaging cell line, GP+E86 (provided by Dr. A. Banks). This cell line has multiple modifications in the packaging elements and substantially reduces the risk of generating replication-competent recombinant virus, which had complicated the previous studies. Several aspects of the bone marrow infection protocol were systematically investigated, including the possible contribution of costimulation of the marrow cells by hematopoietic growth factors during the period of infection. All of the 37 transplanted animals expressed human ADA in their peripheral red blood cells for up to 9 weeks after the transplant. When the hematolymphoid tissues were surveyed by Western analysis and *ADA*-specific polymerase chain reaction (PCR), 68% of the animals were positive at 6 months post-transplant. The percentage of cells bearing the provirus was not uniform in the various tissues examined. This suggested variable infection of the repopulating cells in the different lineages. The addition of a cocktail of growth factors [including interleukin-3 (IL-3), IL-1α, and IL-6] improved the percentage representation of infected cells in the myeloid lineage. This result is consistent with the possibility that stimulation of the cells during infection may improve the infection efficiency of the most primitive repopulating cells but may also affect their subsequent distribution in the transplanted animal.

The role of hematopoietic growth factors in the growth of pluripotent hematopoietic stem cells is being investigated using purified recombinant proteins. A new competitive repopulation assay has been developed to aid in the quantitative analysis of growth factor activities. Two families of inbred FVB strain transgenic mice are being used as donors into parental strain recipient mice. The cells from the transgenic strains are readily distinguishable by Southern analysis. By stimulating cells of one strain during the infection and mixing the cells from each strain prior to infusion into the transplant recipients, it should be possible to analyze the growth factor effects on the long-term repopulating cells.

Initial experiments examining spleen colony-forming unit (CFU-S) progenitors indicate that IL-3, granulocyte colony-stimulating factor (G-CSF), IL-6, IL-7, and leukemia inhibitory factor (LIF) all increase retrovirus infection efficiency. The effect of IL-6 occurs in the absence of a measurable increase in CFU-S number and is consistent with the hypothesis that this factor influences the latency of G_0 to G_1 transition in stem cells. LIF appears to increase CFU-S growth *in vitro* by an effect on a precursor cell. This factor also stimulates the growth of embryonic stem cells, which raises the possibility that the factor directly affects the pluripotent stem cells. A technique for *in vitro* propagation of hematopoietic stem cells would open the way for a variety of gene modification strategies.

Several new vectors bearing immunoglobulin and T cell receptor (TCR) enhancer sequences have been tested. In tissue culture cell lines, no increase in expression was noted in the appropriate lymphoid cell types. A similar result has been observed *in vivo* after bone marrow transplant. The explanation for the failure of the modifications to affect specificity of expression is being investigated.

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II. Characterization of the Moloney Murine Leukemia Virus ψ Region.

Dr. Belmont's laboratory has investigated some of the molecular mechanisms involved in vector assembly. Initial studies have used chemical protection mapping of the RNA in virus particles and phylogenetic analysis to develop a model for the secondary structure of the RNA in the ψ packaging region. Computer analysis of the chemical modification data and primary sequence was conducted using PCFOLD 4.0, which contains algorithms for minimization of free energy. A model of the RNA secondary structure based on all these data proposes that the two strands of RNA are hybridized in a unique homodimer within the 5′ region of ψ. Ongoing studies are now aimed at using site-directed mutagenesis to allow genetic tests of the secondary structure model. It is suspected that the NC peptide encoded in the *gag* gene is involved in the specific recognition of the vector RNA. NC is a prototypic finger protein and is known to have nucleic acid–binding properties. Several vectors for overexpression of NC have been constructed and are being tested. The ability of the free NC peptide to bind viral RNA and thus inhibit the normal packaging process is also being investigated.

III. Use of TCRγ to Analyze T Cell Development.

A major concern for gene therapy research centers on the normal biology of the target cells for gene transfer. Retrovirus vector infection has been used to mark the progeny of hematopoietic stem cells genetically and thus investigate their developmental potential and dynamics. A different approach is being taken to the problem of T cell population kinetics and lineage relationships. In these experiments the rearrangement of the TCRγ loci provides natural markers for families of developing T cells. It is known that Cγ1 begins rearranging early in fetal thymocyte development. Current models suggest that TCRγ loci rearrange in lymphoid progenitors before TCRβ or TCRα. Thus the unique rearrangement, and in particular the specific sequence at the V-J junction in single TCRγs, should mark families of T cells before they clonally diverge with rearrangement of TCRβ. With the help of Dr. Richard Gibbs (Baylor College of Medicine), specific PCR and direct sequencing techniques for Vγ2, Vγ3, and Vγ4 have been developed. Initial experiments have focused on the production of cells bearing rearrangements of Vγ3. Previous data had suggested that cells expressing this gene were produced only during early prenatal thymic ontogeny, that the diversity was very restricted, and that they were anatomically sequestered in the dendritic epidermal cell population. Analysis of adult and developing thymic cells indicates that this receptor can be highly diverse and that the cells expressing this gene are present throughout life. The laboratory is currently using this method to investigate the possible clonal relationships within thymic microanatomic compartments, i.e., thymic nurse cell–enclosed lymphocytes and macrophage rosettes.

Dr. Belmont is also Assistant Professor of Molecular Genetics, Pediatrics, Microbiology, and Immunology at Baylor College of Medicine.

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RETROVIRAL INTEGRATION

PATRICK O. BROWN, M.D., PH.D., *Assistant Investigator*

Work in Dr. Brown's laboratory is focused on the mechanism by which a retrovirus inserts a DNA copy of its genome into a chromosome of its host cell. This integration reaction is an essential step in retroviral replication. Retroviral integration provides a highly efficient means of inserting foreign DNA into mammalian chromosomes and thus has important potential for genetic engineering and gene therapy. Moreover, since integration depends on virally encoded functions and has no known essential cellular counterpart, it is a promising target for development of novel antiviral agents. Two retroviruses are currently being studied: the Moloney murine leukemia virus (MLV) and the human immunodeficiency virus (HIV).

I. Murine Leukemia Virus Integration and Nuclear Entry.

In the past year Dr. Brown and his colleagues have completed a characterization of the structure of intermediates in the MLV integration process. By carrying out the integration reaction *in vitro*, it was possible to identify and recover a key intermediate in the joining of viral to host DNA. In this reaction intermediate, only one strand at each end of the viral DNA molecule is joined to the host DNA. Sequence analysis demonstrated that this initial joint involves the 3′ ends of the viral DNA molecule and the target DNA 5′ ends. The detailed structure of this joint depends directly on the structure of the viral DNA precursor. It was thus possible to show that the linear DNA product of reverse transcription is joined directly to host DNA, without first being circularized, as had previously been believed. The unintegrated linear viral DNA molecule was also characterized. The ends of this molecule are initially blunt but are soon processed by removal of two bases from each 3′ end. This processing event is necessary to expose the specific 3′-OH group that is used in joining the viral DNA to its target. A virus carrying a defective allele of the *int* gene, and thus unable to carry out integration, was shown to be incapable of processing the viral DNA 3′ ends. This result suggests that cleavage of the terminal two bases from the 3′ ends of the linear precursor is one essential role that the Int protein plays in integration. Since this cleavage event is temporally and spatially separated from the joining of viral to target DNA, it is not likely to provide the energy for formation of the new bonds linking viral and target DNA, yet earlier studies by Dr. Brown and his colleagues had shown that no external source of energy is required for integration. It follows that the formation of bonds joining viral and target DNA is probably energetically coupled to cleavage of the target DNA molecule. This hypothesis and the chemical mechanism of the joining reaction are currently under investigation.

Characterization of the native state of unintegrated viral DNA in acutely infected cells has demonstrated that the DNA is in a large (160 S) nucleoprotein complex that includes all the activities required for efficient integration. This complex, with its integration activity intact, can be efficiently immunoprecipitated using antisera specific for the viral capsid protein. Moreover, while the viral DNA in the complex can be cut with restriction endonucleases, the resulting fragments remain associated with a 160 S particle. These data suggest that this intracellular form of the virus has significant similarities to the core of the extracellular virus particle. Further studies directed at the identification of the components of this complex and definition of its architecture are under way. It is likely that this nucleoprotein complex has important roles in addition to its role in integration. For example, it may play an active part in directing the transport of the viral genome into the nucleus of the infected cell. Studies that focus on the mechanism by which the viral nucleoprotein complex gains access to the nucleus are currently in progress in Dr. Brown's laboratory.

II. HIV Integration.

HIV is unique among the retroviruses both in its importance as a cause of human disease and in the complexity of its life cycle. Dr. Brown and his colleagues have developed methods for studying the integration of HIV in a cell-free system and have begun the biochemical characterization of this process. Preliminary experiments have established the conditions necessary for activity and shown that the *in vitro* reaction can be carried out in the absence of an extrinsic energy source and that the enzymatic machinery required for HIV integration is in a nucleoprotein complex with the viral DNA. Further characterization of the molecular mechanism of

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HIV integration is in progress, as are efforts to develop a rapid assay suited to large-scale screening for inhibitors of integration.

Dr. Brown is also Assistant Professor of Pediatrics and of Biochemistry at the Stanford University School of Medicine.

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CREATING MICE WITH SPECIFIC MUTATIONS BY GENE TARGETING

MARIO R. CAPECCHI, PH.D., *Investigator*

Homologous recombination between DNA sequences residing in the chromosome and newly introduced, cloned DNA sequences (i.e., gene targeting) allows the transfer of any modification of the cloned gene into the genome of a living cell. Furthermore, if the recipient cell is a pluripotent, mouse embryo-derived stem (ES) cell, it is possible to transfer that modification (created in a test tube) to the germline of a living mouse. Thus the potential exists for the defined modification of any mouse gene and evaluation of the phenotypic consequences of that modification. The ability to generate specific mouse mutations via gene targeting should have a major impact on many phases of mammalian biology, including development, cancer, immunology, neurobiology, and human medicine.

I. Disruption of the *hprt* Gene.

Mammalian cells can mediate recombination between homologous DNA sequences, but they demonstrate an even greater propensity for mediating nonhomologous recombination. Identification of homologous recombination events in a vast pool of scattered, nonhomologous recombination events is the resulting problem. The *hprt* (hypoxanthine phosphoribosyl transferase) gene is an ideal model system for developing the technique of gene targeting in ES cells, because the targeting event can be selected directly. Since this gene is on the X chromosome, only one mutant copy is needed to yield the recessive $hprt^-$ phenotype in male ES cells. The $hprt^-$ cells are selected by growth in the presence of the base analogue 6-thioguanine (6TG), which kills $hprt^+$ cells.

Two classes of targeting vectors were tested for their ability to disrupt the *hprt* gene: sequence replacement and sequence insertion vectors. With yeast as a paradigm it was anticipated that sequence replacement vectors would replace endogenous DNA with exogenous sequences, whereas sequence insertion vectors would insert the entire vector DNA sequence into the endogenous locus. Each class of vectors contains a neomycin resistance (neo^r) gene within an exon of *hprt*. This arrangement not only disrupts the coding sequence of *hprt* but also provides a selectable marker for cells containing an integrated copy of the recombinant vector (resistance to the drug G418).

After the introduction of the targeting vectors into ES cells by electroporation and selection for resistance to G418 and 6TG, all survivors were found to have lost *hprt* activity as a result of targeted disruption of the *hprt* gene. Replacement vectors and insertion vectors were equally efficient at disrupting the endogenous *hprt* gene. Furthermore, both vectors showed the same strong dependency of the targeting frequency on the extent of homology between the targeting vector and the endogenous DNA sequence. Over the range tested (2.9–14.3 kb) a 5-fold increase in DNA sequence homology resulted in approximately a 100-fold increase in the targeting frequency.

II. Nonselectable Genes.

The *hprt* gene was chosen as a model in the initial studies because direct selection could be used to isolate cells in which a homologous recombination event had occurred. However, in the vast majority of cases a selectable cellular phenotype is not associated with the inactivation of both copies of a gene or with the more frequent single-copy inactivation event. Therefore it is desirable to have some means for identifying the rare ES cell in which a nonselectable gene has been inactivated. This can be achieved by using indirect enrichment and/or screening procedures. Pursuit of enrichment procedures rather than screening procedures was chosen because, if successful, they should be less labor intensive and permit identification of rarer events.

Recently an enrichment procedure was described that is independent of the function of the target gene and of its expression in ES cells. This positive-negative selection (PNS) procedure uses a positive selection for cells that have incorporated the targeting vector anywhere in the ES genome and a negative selection against cells that have randomly integrated the vector. The net effect is to enrich for cells containing the desired targeted mutation.

PNS was used to enrich for ES cells containing disruptions of the *hprt* gene. After introduction of the *hprt*-PNS-targeting vector into ES cells, virtually all (19/24) of the selected colonies contained targeted disruptions of the *hprt* gene, even though direct selection for the $hprt^-$ phenotype was not done.

III. The Mouse *box* Genes.

Recently molecular genetic analysis of early development in *Drosophila* has revealed a network of genes that control the formation of its metameric pattern. Many of these genes share a DNA se-

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quence, the homeobox domain, that encodes a protein DNA binding motif. On the basis of DNA sequence similarity, a set of homeobox-containing genes, the *box* genes, have been isolated in the mouse. The function of these genes is not known. However, the embryonic expression patterns of these genes imply roles in establishing positional information during development. Twenty-five mouse homeobox-containing *Antennapedia* genes have been isolated and shown to be contained in four linkage groups: *box1, box2, box3,* and *box5.* PNS was used to disrupt seven of these genes in ES cells. These cells have, in turn, been used to generate mouse chimeras and will be evaluated for their ability to transmit the mutant allele to their progeny. Targeted disruption of these genes should not only reveal the phenotypes associated with the inactivation of the individual genes but, also through epistasis and molecular analysis, help define the developmental network controlling early mouse morphogenesis.

IV. *int*-related Genes.

In addition to disrupting genes that encode transcription factors that activate the progression of the developmental program of the mouse, the laboratory is also focusing on genes that mediate cell-cell interactions that feed back information to the program concerning the localized developmental progress achieved within the embryo at any given stage.

The *int*-related genes are excellent candidates for this second class of genes. These genes were first identified as sequences activated in mammary tumors of mice by the nearby insertion of the mouse mammary tumor virus. The protein products show sequence similarities to growth factors. *In situ* hybridization analyses reveal diverse but highly restricted patterns of expression during development. Four *int* genes have been identified: *int-1* to *int-4.* The *int-2* homologue in *Drosophila*, *wingless*, participates in establishing cell identity within segments via cell-cell interactions by indirectly modulating the activity or quantity of transcription factors involved in the developmental program. Transplantation experiments in chick embryos suggest a role for the *int-2* product in inducing neighboring cells to progress along a specific differentiation pathway. The above properties are consistent with a role for *int-1* and *int-2* in cell-cell interactions via unidentified receptors. At the same time, they may indirectly modulate the activities of transcription factors and thereby direct the developmental program along specific pathways. PNS was used to disrupt the endogenous *int-1* and *int-2* genes in ES cells. These cell lines were also used to generate mouse chimeras, which will be evaluated for transmission of the mutant alleles to their progeny.

The power of gene targeting is that the experimenter chooses both which gene to mutate and how to mutate it. The precision afforded by gene targeting should allow the formulation of genetic questions with sufficient clarity to yield informative answers.

Dr. Capecchi is also Professor of Biology at the University of Utah and Professor of Human Genetics at the University of Utah School of Medicine.

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HUMAN GENETICS AND MUTATIONAL MODELS

C. Thomas Caskey, M.D., *Investigator*

I. Lesch-Nyhan Syndrome.

The complete DNA sequence of the hypoxanthine guanine phosphoribosyltransferase (HPRT) gene has been determined by automated sequencing and computer analysis. The method utilized "shotgun" sequencing, a method with the advantage of sequence redundancy and accuracy, simplicity, and rapid data analysis. One continuous sequence of 58,000 base pairs (bp) was achieved by sequencing 250,000 bp. This sequence identified a new class of minisatellite sequence within the HPRT gene with considerable polymorphism. The sequence permitted development of a rapid polymerase chain reaction (PCR) multiplex (many genetic sites) and simple nonradioactive method to identify mutations causative of Lesch-Nyhan (LN) syndrome. Automated DNA sequencing of these mutations makes possible identification of the new mutations in the germline causing LN in a specific family. These studies are now completed in 1–2 days rather than 7–14 days by earlier methods. Application to the prevention of disease by female carrier detection and prenatal diagnosis has been successfully achieved for 32 families.

II. Uricase.

Humans are devoid of uricase activity and eliminate purines as uric acid, a cause of gout in some patients. The mouse possesses uricase activity and eliminates purines as allantoin. HPRT deficiency in humans leads to LN syndrome, while the deficiency in mice is without phenotypic effects. It is possible that uricase has a protective effect on HPRT-deficiency central nervous system damage. The uricase cDNA and gene of mice are characterized. A nonfunctional gene in humans has been cloned. The functional cDNA of the baboon has been cloned and characterized. All have been compared. Two nonsense mutations were found in the human sequence that would render it nonfunctional. The highly conserved nature of the human, mouse, and baboon coding sequence suggest the mutation in humans is very recent. Attempts to create the uricase$^-$/HPRT$^-$ mouse are now proceeding, using the embryonic stem cells of the mouse and insertional mutagenesis via homologous recombination.

III. Duchenne Muscular Dystrophy.

Duchenne muscular dystrophy (DMD) is a severe X-linked muscular dystrophy that occurs by new mutations, which are largely (85%) deletions and duplications of portions of the 2.5 million bp gene. A simple nonradioactive scanning DNA detection method has been developed and validated by an international multicenter collaboration. The PCR multiplex method uses DNA sequence information on the DMD gene mutation "hot spots" (prone to deletion and duplication) and presently includes nine positions. This collaborative study found the method capable of diagnosing 80% of all gene duplications and deletions. Fluorescent detection methods have made quantitation and application to detection of female carriers possible. This simple diagnostic approach for DMD is now the standard of practice for diagnosis.

The identification of the mutations in the DMD gene of mice that cause *mdx* phenotype is under way. This is made possible by the complete cloning and sequencing (75%) of the mouse dystrophin cDNA. The *mdx* mutation in the Bulfield mutant is a nonsense mutation at a position early in the gene. The two *mdx* mutants developed in collaboration with Dr. Verne Chapman by germline mutation are under study for their mutation.

IV. Ornithine Transcarbamylase Deficiency.

Ornithine transcarbamylase (OTC) deficiency is the most common urea cycle disorder in humans and leads to severe neonatal coma and death. A new rapid scanning method, which is based on knowledge of the OTC DNA sequence, has been developed for the mutations. Mutations are detected by cleavage at the site following heteroduplex formation (wild-type sequence:mutant sequence) and chemical cleavage. The approach has the advantage of speed, ease, and scanning over large DNA segments. The unique mutations of seven families were identified, and this knowledge was used to diagnose female carriers and conduct prenatal diagnosis.

Genetic correction of the OTC-deficient *sparse fur* (*spf*) mouse has been achieved by transgenic approaches. A recombinant transgene consisting of the human OTC cDNA under the control of an 800 bp mouse promoter was used; single-cell embryos

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(*spf*) were injected, and those newborn mice were found to be corrected from the *spf* disease traits. Analysis of these transgenic mice found the correcting transgene to be expressed in the small bowel, not in the liver. These studies suggest delivery of correcting genes to small bowel may be a more facile method of gene correcting efforts than that directed to correction in liver. Viral vectors are now developed toward that goal.

V. Adenosine Deaminase.

Long-term expression of human adenosine deaminase (ADA) has been obtained in mice via the infection of hematopoietic stem cells with an N2-derivative retroviral vector (see the report of Dr. John W. Belmont). An efficient system of retroviral gene transfer of ADA in human hematopoietic cells is now being developed. Five to 20% of unselected human colony-forming progenitors contained an integrated copy(ies) of the provirus after infection with virus-containing supernatants. Infected bone marrow cells (BMCs) were detected for up to seven weeks in long-term cultures. Transduced ADA expression was also detectable after infection of BMCs from two ADA-deficient patients. In parallel, modified vectors have been constructed for high-level expression in T and B cells. These produce high titers of virus and successfully transduce human ADA in target cell lines. Their potential tissue-specific expression is being evaluated in cell lines and in long-term reconstituted animals. These studies provide a rational scientific basis for attempts to correct ADA immunodeficiency in humans.

VI. Xq28 Disease Genes.

Cloning and characterization of the terminal end of the long arm of the human X chromosome has the potential for simultaneous identification of 25 disease genes. Yeast artificial chromosome (YAC) libraries have been prepared from somatic cell hybrid lines containing either an intact X chromosome (4.12) or Xq24-qter (X3000-11), and human clones have been identified. Over 200 human clones have been isolated from the X3000-11 library, and 40 have been localized to specific X regions. One of these YAC clones appears to contain the gene defective in the oculocerebrorenal syndrome of Lowe. The 4.12 library has over 150 human clones, and 20 have been localized to specific X regions. A new method of preparing human-specific DNA from the YAC (*Alu* PCR) uses PCR amplification of human DNA specifically with oligonucleotide primers directed to the human *Alu* repetitive sequence. This method allows isolation and characterization of human DNAs from somatic cell hybrids, preparation of insert sequences without subcloning, and direct isolation of identifying tag sequences from the YAC 5′ and 3′ sequences. A means of identifying expressed genes in a chromosomal region has been developed using rodent/human somatic cell hybrids containing small regions of the human X by the direct isolation of human cDNA from heterogeneous nuclear RNA.

VII. Peptide Chain Termination.

The isolation and characterization of a mammalian release factor (RF) cDNA has been the focus of recent efforts in protein synthesis. The rabbit RF cDNA has been fully characterized and expressed as a protein in bacteria. The *in vitro* synthesized RF has stop codon specificity and requires GTP, all features of a mammalian RF. Sequence comparisons find the RF to be similar to tryptophanyl-tRNA synthetases from bacteria and yeast, suggesting an evolutionary relationship between mammalian RFs and the AA-tRNA synthetases. The characterization of the mouse RF gene for function domains is under way. Structure-function comparisons of RF genes from a variety of bacterial species and mammalian mitochondria are proceeding.

Dr. Caskey is also Professor at the Institute for Molecular Genetics and Professor of Medicine, Biochemistry, and Cell Biology at Baylor College of Medicine.

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RNA CATALYSIS AND THE STRUCTURE OF CHROMOSOME ENDS

THOMAS R. CECH, PH.D., *Investigator*

The goal of Dr. Cech's laboratory is to contribute to the understanding of gene expression and chromosome structure in eukaryotes. In the area of gene expression the focus is on RNA splicing, particularly self-splicing, in which the folded structure of the RNA catalyzes the rearrangement of phosphodiester linkages. The ribosomal RNA intron from the unicellular eukaryote *Tetrahymena thermophila* provided the first example of RNA self-splicing and continues to be the major experimental system in Dr. Cech's laboratory. The objective is to understand both the mechanism of RNA catalysis and the structure of the active site of the molecule.

In the area of chromosome structure, DNA-protein interactions at telomeres, the natural ends of linear chromosomes, are being studied. *Oxytricha nova*, another protozoan, is particularly rich in telomeres, with ~1 million-fold more chromosomes per nucleus than a human cell. This has facilitated the purification of the telomere-binding protein. The aim is to understand first how the protein recognizes the repeated T_4G_4 sequence at the chromosome ends and then how the protein interacts with the DNA replication machinery. Human telomeres are similar at the DNA level, consisting of a repeated T_2AG_3 sequence; thus the findings in *Oxytricha* may be generally applicable.

I. RNA Catalysis.

A. *Reverse self-splicing of the* Tetrahymena *intron.* Incubation of ligated exon RNA with the linear intron produced a molecule in which the splice-site sequences of the precursor RNA were re-formed. Integration of the intron into ligated exon substrates that have the ability to form stem-loop structures was reduced at least one order of magnitude over short, unstructured substrates. This led to the proposal that the formation of such structures helps drive splicing, an intrinsically reversible reaction, in the forward direction. Integration of the *Tetrahymena* intron into a β-globin transcript also occurred *in vitro*; this result has implications for transposition of group I introns.

B. *Stereochemistry of RNA cleavage by the* Tetrahymena *ribozyme.* Shortened versions of the *Tetrahymena* intron act as RNA enzymes, or ribozymes. One of the ribozyme activities is that of a sequence-specific endoribonuclease. This system facilitates study of the chemistry of the reaction. A single phosphorothioate was introduced at the cleavage site in the substrate RNA. Product analysis revealed that the reaction proceeds with inversion of configuration at phosphorus, consistent with an in-line, S_N2 (P) mechanism. Thus the ribozyme reaction is in the same mechanistic category as the individual displacement reactions catalyzed by protein nucleotidyltransferases and nucleases.

C. *Fidelity of RNA cleavage by the* Tetrahymena *ribozyme.* Specificity of cleavage is determined by base-pairing between the active site of the ribozyme and its RNA substrate. Surprisingly, single-base changes in the substrate RNA that give a mismatched substrate-ribozyme complex enhance the rate of cleavage. The mechanistic explanation is that mismatches accelerate a rate-limiting product-release step. Addition of a destabilizing agent (urea or formamide) reverses the substrate specificity, allowing the ribozyme to discriminate against mismatched substrates.

D. *Defining the inside and outside of the ribozyme.* Fe(II)-EDTA, a solvent-based reagent that cleaves both double- and single-stranded RNA, was used to investigate the structure of the *Tetrahymena* ribozyme. Most of the catalytic core is protected from cleavage. The data provide experimental evidence that an RNA enzyme, like a protein enzyme, has an interior and an exterior. The technique is expected to be informative for probing the tertiary structure of RNA molecules.

II. DNA-Protein Interactions at Telomeres.

A. *Properties of the protein.* Glycerol gradient sedimentation and protein-protein crosslinking indicated that the 43 and 55 kDa polypeptides are subunits of a heterodimer. Both subunits are very basic (pI > 8.5).

B. *Assembly and self-association of telomeric complexes.* The protein was found to bind to the 3′ end of single-stranded oligonucleotides that have the telomeric sequence $(T_4G_4)_n$, where $n > 1$, reconstituting the methylation protection seen with macronuclear DNA. Three oligonucleotide-protein complexes were resolved by nondenaturing gel electrophoresis; all were specific for the telomeric

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DNA sequence. The complexes also differed in their protein-DNA contacts and in the rate of DNA exchange. The patterns of protein-DNA interaction suggested a model in which the protein can bind either to the two repeats at the 3′ end of the DNA or to two internal repeats; only in the latter case can it make an additional set of contacts to form the more stable complex. Native telomeric chromatin isolated from *Oxytricha* contains both types of complexes. The reconstituted monomeric complexes associated to give a high-molecular-weight form with an altered chemical footprint. Such interactions are proposed to mediate the association of chromosome ends *in vivo*.

Dr. Cech is also American Cancer Society Research Professor of Chemistry and Biochemistry and of Molecular, Cellular, and Developmental Biology at the University of Colorado at Boulder and Professor of Biochemistry, Biophysics, and Genetics at the University of Colorado Health Sciences Center.

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GENOMIC SEQUENCING

GEORGE M. CHURCH, PH.D., *Assistant Investigator*

Several laboratories are sequencing small genomes (1–15 Mbp) from each phylogenetic kingdom. Comparisons of these sequences will define consensuses for most classes of protein domains, evolutionary conservation and evolutionary change. Up to 20-fold higher substitution rates in nonconserved compared with coding nucleotides allow the discrimination of random open reading frames from those encoding proteins that confer a selective edge. Operon and regulon gene organization can reveal physiological relationships and hence contribute clues to possible functions for the newly discovered genes. The genome closest to completion is *Escherichia coli*, with 20% of its 4.7 Mbp completed by 2,000 biologists. Toward the goal of completing the *E. coli* and *Salmonella typhimurium* genome sequences, over 1,700 films have been produced in the past year by methods described below (see also *Science* 240:185–188). About 10% have been digitally scanned and proofread using the sequencing reading and assembling software REPLICA. In collaboration with Drs. Ken Rudd (Food and Drug Administration) and Jim Ostell (National Library of Medicine) and co-workers, an *E. coli* genetic map/restriction map/DNA sequence database has been created.

I. New DNA-sequencing Methods.

In multiplex DNA sequencing, 480 sequencing reaction sets, each tagged with specific oligonucleotides, are run on a single gel in 12 pools of 40 and transferred to a membrane. Up to 75 such membranes are hybridized simultaneously. The resulting sequence images are digitized and sequence interpretations are superimposed on the enhanced two-dimensional images for editing. The computer program (REPLICA) uses internal standards from multiplexing to establish lane alignment and lane-specific reaction rules by discriminant analysis. Images with overlapping data can be viewed side by side to facilitate decision making, by integrating automatic base assignment routines with high-resolution image display and interactive multisequence alignments.

II. Specific Genomic Regions.

Genomic regions of special interest in many cases may be easily included in large-scale projects. These regions have been selected by genetic complementation, DNA-protein interactions, or subtractive hybridization. The largest contiguous sequence obtained by the above methods is 18 kbp (from 140 kb of raw data) covering the *Salmonella* cobalamin biosynthesis operon originally selected by complementation (in collaboration with Dr. John Roth). Regions of putative DNA-protein interactions have been cloned *en masse* by virtue of differential enzyme cutting and sequenced. One site studied in detail appears to be responsive to pyrimidines. Those few regions conserved between two distant genomes or different between two closely related genomes may be selected out of the whole by a few rounds of differential hybridization. Work has begun to study the genomic sequence differences between pathogenic and nonpathogenic strains of *E. coli* and *Shigella* (in collaboration with Drs. Donald Straus and Fred Ausubel).

Dr. Church is also Assistant Professor of Genetics at the Harvard Medical School.

MOLECULAR GENETICS OF HUMAN DISEASE

FRANCIS S. COLLINS, M.D., PH.D., *Associate Investigator*

Dr. Collins's laboratory is involved in four major projects that involve the molecular basis of human disease. Three of these projects involve the identification of genes that cause disease but whose protein products have not been possible to characterize directly. With the new technique of reverse genetics, much progress has been achieved.

I. Cystic Fibrosis.

Intense efforts over the last four years culminated in the successful cloning of the cystic fibrosis (CF) gene in the late summer of 1989, in collaboration with Drs. Lap-Chee Tsui and Jack Riordan (Hospital for Sick Children, Toronto). A region of ~500,000 bp was cloned, using a combination of chromosome jumping and chromosome walking. Genetic analysis of a large number of CF families indicated the most likely position of the CF mutation; a survey of that region for conserved and/or transcribed sequences yielded a genomic fragment of DNA that proved to be the 5′ end of the CF gene. The complete cDNA sequence has been obtained. This sequence encodes a protein of ~168 kDa, which has homologies to the multidrug resistance gene and a variety of other genes for transporter pumps of lower organisms. Specifically, the protein product contains several hydrophobic domains that presumably anchor it to the cell membrane. It also contains a region that is probably an ATP-binding site, which indicates that the protein may be an ATP-activated pump. The proof that this represents the CF gene is the identification of a 3 bp deletion in exon 10 that occurs in 70% of CF chromosomes but is never found in normal chromosomes. This deletion results in the loss of a single phenylalanine residue in the protein product, which is located in the ATP-binding domain.

This achievement represents the first time that a gene has been cloned by reverse genetics without the availability of gross chromosomal rearrangements. The door is now open to a large number of experiments that may lead to an understanding of the basic defect in CF. Current priorities include the identification of the other mutations responsible for CF, which undoubtedly will affect the same gene, and the development of a gene transfer system to show that expression of the normal version of this gene is capable of correcting the defect in CF cells. Dr. Collins and his colleagues have named the gene CFTR (cystic fibrosis transmembrane regulator). (See *Science* 245:1058–1065, 1066–1073, and 1073–1080 for details of the cloning.)

II. Neurofibromatosis.

Neurofibromatosis (NF1) is a common autosomal dominant human disease characterized by the development of multiple benign tumors and an increased risk of cancer. Considerable progress has been made in identifying the NF1 gene, which had been previously mapped to chromosome 17 by linkage analysis. DNA samples from two patients who have NF1 in association with balanced translocations involving 17q11.2 have been used to narrow the location of the NF1 gene to a region of ~500 kb. This achievement was made possible by cloning a large number of *Not*I linking clones from chromosome 17 and testing them against pulsed-field gel electrophoresis (PFGE) blots from these two patients. A probe was eventually identified, denoted 17L1, which lies <400 kb away from both translocation breakpoints. This region is being investigated for candidate genes, in collaboration with Dr. Raymond L. White (HHMI, University of Utah).

III. Huntington Disease.

The Collins laboratory is an active participant in the collaborative effort to identify the Huntington disease gene on the short arm of chromosome 4. This genetic analysis, primarily carried out by Dr. James Gusella, has led to conflicting information about the precise location of the gene. Current efforts are focused on applying the chromosome jumping technique to fill in the gaps in the physical map of the terminal 6 million bp of 4p, as well as constructing high-quality cDNA libraries from various regions of the human brain to search for candidate transcripts.

IV. Hemoglobin Switching.

The switch from production of fetal hemoglobin to adult hemoglobin at about the time of birth in the human provides an interesting model system for understanding the developmental regulation of gene expression and has the additional importance of potential clinical utility: if this switch could be

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reversed in individuals with sickle cell anemia or thalassemia, their disease could be cured. The laboratory has been investigating DNA sequences in the promoter of the fetal globin gene in an effort to understand this developmental switch. A series of seven nuclear protein factors that bind in a sequence-specific manner to the promoter have been identified, and a number of these have been partially purified using affinity columns. The role of the transcription factor Sp1 in the γ-globin promoter has also been carefully investigated; Sp1 has been found to be responsible for much of the transcriptional activity associated with the CACCC sequence, a control sequence common to nearly all globin genes. A different nuclear protein that binds to this CACCC sequence has recently been cloned and is under active investigation. Another approach is an evolutionary comparison of prosimian and primate γ promoter sequences, to address the question of how the γ-globin gene became recruited as a fetal gene, since it previously functioned in an embryonic pattern. Finally, the mutations identified in the γ promoter that cause hereditary persistence of fetal hemoglobin are beginning to be understood; at least three of them show significant alterations in binding of one or more of the nuclear proteins identified, which suggests a mechanism for their phenotype. These studies should improve understanding of the mechanism of fetal globin regulation and may lead to new ideas about therapy.

Dr. Collins is also Associate Professor in the Departments of Internal Medicine and Human Genetics at the University of Michigan Medical School.

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MOLECULAR ANALYSIS OF RNA POLYMERASE II

JEFFRY L. CORDEN, PH.D., *Associate Investigator*

Dr. Corden's laboratory has continued to study RNA polymerase structure and function. During the past year attention has been focused on the unusual repeated sequence at the carboxyl terminus of the largest subunit of mouse RNA polymerase II (RPII). This domain consists of 52 repeats of a seven–amino acid sequence with the consensus sequence Tyr-Ser-Pro-Thr-Ser-Pro-Ser. A similar carboxyl-terminal domain (CTD) is found in the largest subunit of RPII from a variety of species, but it is not present in eukaryotic RNA polymerases I or III or in prokaryotic RNA polymerases, indicating that it plays a unique role in RPII transcription. Ongoing studies in Dr. Corden's laboratory are designed to elucidate the function of this domain.

One characteristic of the CTD is a high content of amino acids that can be modified by phosphorylation. Actively transcribing RPII is highly phosphorylated; analysis of breakdown products of the largest subunit suggested that the CTD was the primary site of phosphorylation. One objective of Dr. Corden's laboratory has been to identify and analyze the protein kinase that phosphorylates the CTD. The first step was to show that short peptides containing several of the seven–amino acid consensus blocks were phosphorylated in whole-cell extracts from mouse cells. Preliminary characterization showed that this kinase activity phosphorylates serine residues in the CTD. Both ATP and GTP can serve as phosphate donors. With this peptide substrate assay, a protein kinase that phosphorylates the CTD was purified from mouse ascites tumor cells.

The purified enzyme contains subunits of 58 and 34 kDa. The 34 kDa subunit was isolated and digested with trypsin, and several peptides were purified and sequenced. Comparison of p34 peptide sequences to protein sequence data banks revealed that p34 is the mouse homologue of the yeast cell-cycle control protein cdc2. The cdc2 protein kinase and its budding yeast homologue CDC28 have been implicated in the transitions between G1 and S phase and between G2 and M phase of the cell cycle. The discovery that cdc2 is part of a complex that can phosphorylate RPII suggests that phosphorylation of the CTD may play some role in cell-cycle control and that transcription may be regulated in a cell-cycle-dependent fashion. Another implication of the involvement of cdc2 in transcriptional regulation is that CTD kinase may be part of the signal transduction pathway that links cellular responses at the cell surface to changes in gene expression. The cdc2 protein kinase is itself a phosphoprotein and may be phosphorylated by proto-oncogene products like c-*src* and c-*mos*. Therefore changes in the signal transduction pathway brought about by oncogenic transformation may exert their effects on gene expression through CTD kinase. Current studies are aimed at characterizing the CTD kinase subunits and developing *in vivo* and *in vitro* systems for studying the role of CTD phosphorylation in the transcription process.

One approach has been to purify the CTD and use the detached domain in biochemical experiments designed to look for interactions between this domain and components of the transcriptional apparatus. The CTD has been overexpressed in both prokaryotic and eukaryotic expression vector systems, and the resulting proteins have been purified. The CTD expressed in eukaryotic cells is highly phosphorylated. The structures of both the phosphorylated and unphosphorylated CTDs and their interactions with other transcription components are being analyzed.

A genetic approach developed in Dr. Corden's laboratory enables the reintroduction by transfection of RPII genes with alterations in the CTD coding sequence. This approach has been made possible by cloning and characterizing α-amanitin–resistance alleles of the gene of the largest RPII subunit. Such amanitin-resistance genes can be transfected into tissue culture cells, where they confer resistance to amanitin. Secondary mutations in the CTD of the amanitin-resistance gene can be coselected, and its effect on the ability to confer amanitin resistance can be tested. This approach has shown that large deletions in the CTD are lethal, implying that the CTD plays an essential role in the transcription process. Smaller deletions and point mutations have recently been constructed and reintroduced into cells. The effects of these mutations on the ability of RPII to transcribe properly are being analyzed.

In collaboration with the laboratory of Dr. Paul A. Overbeek (HHMI, Baylor College of Medicine), transgenic mice harboring α-amanitin–resistant RPII were recently developed to analyze mutations in RPII. These mice are resistant to high concentrations of amanitin; under these conditions transcrip-

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tion is carried out by the transgenic RPII. Similar mice with deletions in the CTD are being analyzed for defects in the transcription process.

Dr. Corden is also Associate Professor in the Department of Molecular Biology and Genetics at The Johns Hopkins University School of Medicine.

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TRANS-REGULATION OF HIV-1 GENE EXPRESSION

BRYAN R. CULLEN, PH.D., *Assistant Investigator*

Human immunodeficiency virus type 1 (HIV-1) is the predominant etiologic agent of acquired immune deficiency syndrome (AIDS). The disease AIDS may be viewed as the end stage of an extended, chronic infection of the human host by the HIV-1 retrovirus. After the burst of viral replication that accompanies initial infection, HIV-1 replication in the host becomes relatively quiescent. During this phase, which may be prolonged, low levels of viral replication appear to occur in cells that can act as a reservoir of HIV-1 infection, including various cells of the macrophage/monocyte lineage. This phase appears to be associated with a gradual decline in the $CD4^+$ T cell count. Initial detectable disease eventually manifests itself as a less-severe immune deficiency, AIDS-related complex (ARC). Subsequent deterioration of the immune system then appears inexorable, leading finally to viremia and a dramatic depletion in the remaining $CD4^+$ population. Clinical deterioration, marked by severe opportunistic infections and central nervous system degeneration, then ensues, finally leading to patient death.

The replication of HIV-1 in the infected host may be seen to follow a complex, chronic course. This pattern is somewhat similar to that noted for the related animal retroviruses of the lentivirus type but is distinct from the acute replication typical of the majority of retroviruses, such as the avian leukemia virus (ALV) and murine leukemia virus (MLV). The chronicity of HIV-1 replication in the host may imply a complex balance between virus replication and host antiviral response. At least part of the ability of HIV-1 to achieve this balance may result from the complexity of its genetic makeup. Like other lentiviruses, but unlike ALV or MLV, HIV-1 encodes nonstructural proteins, which act in trans to modulate viral gene expression. Functional expression of at least two of these, Tat and Rev, has been shown to be essential for viral replication *in vitro*. It is the aim of Dr. Cullen's laboratory to understand how the expression of the genome of HIV-1 is regulated and, in particular, to delineate fully the roles and mechanisms of action of the HIV-1 Tat and Rev trans-activators.

The HIV-1 Tat trans-activator acts to enhance greatly the expression of both viral and heterologous sequences linked to the viral long terminal repeat (LTR) promoter element. Dr. Cullen's laboratory has presented evidence suggesting that Tat exerts this effect via a bimodal mechanism, resulting in both an increase in the rate of transcription of HIV-1 LTR-specific mRNAs and an enhancement in the utilization of those mRNAs by the host cell translational machinery. The Tat protein was, however, also shown to be localized predominantly to the nucleus of expressing cells, thus arguing against a direct role for Tat in influencing viral mRNA translation. The target sequence for Tat action, the trans-activation response (TAR) element, has been mapped to a transcribed region of the HIV-1 LTR sequence immediately adjacent to the site of transcription initiation. This region has the potential to form a highly stable RNA stem-loop structure. Current evidence suggests that mutations that affect the integrity of this RNA structure also prevent Tat-mediated trans-activation of HIV-1 gene expression. The possibility therefore exists that Tat may affect HIV-1 gene expression via an RNA-mediated, rather than DNA-mediated, TAR sequence recognition event. This putative protein-RNA interaction may be the key to at least an initial understanding of the mechanism of action of Tat.

Although the *tat* gene product acts to enhance greatly the level of expression of any gene linked to HIV-1 LTR, the viral *rev* gene product acts post-transcriptionally to modulate specifically the expression of virally encoded mRNAs. In the absence of Rev, the incompletely spliced viral mRNAs that encode the virion structural proteins Gag and Env are synthesized at normal levels but excluded from the cell cytoplasm. They cannot, therefore, be functionally expressed. The HIV-1 Rev protein has been shown to induce the nuclear export of these viral mRNAs and therefore is essential for virion synthesis. A specific target sequence for Rev function, the Rev response element (RRE), has been identified and shown to coincide with a highly stable, predicted RNA secondary structure contained within the HIV-1 *env* gene. Evidence suggests that this sequence specificity is directly encoded within the Rev protein itself. The HIV-1 *rev* gene product is therefore the first eukaryotic regulatory protein that has been shown to regulate mRNA transport in a sequence-specific manner.

A mutational analysis of the HIV-1 *rev* gene conducted in Dr. Cullen's laboratory has provided initial evidence for the existence of two functional domains within the HIV-1 Rev trans-activator equiv-

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alent to the binding and activation domains observed in some eukaryotic transcriptional trans-activators. Targeted mutagenesis of the putative activation domain gave rise to Rev proteins that displayed a dominant negative phenotype. These mutant *rev* genes may have potential use in a novel gene therapy approach to AIDS treatment that has been referred to as intracellular immunization. This possibility, which is being aggressively pursued by Dr. Cullen's laboratory, is an example of the generalization that basic research in the biomedical sciences can lead to unexpected discoveries of direct medical significance.

Dr. Cullen is also Assistant Medical Research Professor in the Departments of Medicine and of Microbiology and Immunology at Duke University Medical Center.

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MOLECULAR MECHANISMS IN LYMPHOCYTE DEVELOPMENT

STEPHEN V. DESIDERIO, M.D., PH.D., *Assistant Investigator*

The development of lymphocytes, the principal effector cells of the immune system, occurs in two phases. The first phase culminates in the deployment of antigen receptors on the cell surface. The second phase, encompassing activation, growth, and terminal differentiation, is triggered in part by specific interactions between antigens and their receptors. Molecular mechanisms that underlie both phases of immune development remain the focus of work in the laboratory.

I. Molecular Mechanism of Antigen Receptor Gene Assembly.

The variable regions of immunoglobulin (Ig) and T cell receptor (TCR) chains are encoded in separate germline DNA segments that are brought together by site-specific recombination. Rearrangement is mediated by recombinational signal sequences—conserved heptamer and nonamer elements that are separated by a spacer region. The spacer regions fall into two classes of 12 and 23 base pairs (bp); recombination normally occurs only between gene segments carrying spacers of different lengths. Previous work in this and other laboratories identified B lymphoid cell lines that undergo continuing rearrangement of Ig gene segments during propagation in culture. Dr. Desiderio's laboratory subsequently developed a direct physical assay for the rearrangement of exogenous, integrated recombinational substrates in these cell lines; the assay has been used to examine the molecular details of the recombination reaction and its DNA sequence requirements. In conjunction with this approach, the laboratory has undertaken to isolate components of the recombinational apparatus by identifying and purifying proteins that interact specifically with recombinational signal sequences.

A. *An alternative pathway of Ig gene rearrangement: mechanistic and immunologic implications.* The assay for *in vivo* rearrangement of model substrates relies on direct observation of the products of recombination and places few constraints on the structures of the recombinants. This enabled Dr. Desiderio and his co-workers to discover a pathway for antibody gene rearrangement with important mechanistic and immunologic implications. This unusual reaction, which occurs at 1/10 the frequency of normal joining, results in the fusion of the recombinational signal sequences of one gene segment to the coding sequence of another, resulting in signal sequence replacement. This observation led Dr. Desiderio and his colleagues to propose that in the recombination reaction the initial pairing and cleavage of Ig and TCR gene segments yields an intermediate, in which four free DNA ends (two coding ends and two flanking ends) are held in proximity. Commonly this intermediate would be resolved by joining of the coding ends; less frequently the coding sequences of one segment are joined to the flanking sequences of the other, resulting in signal sequence replacement. Signal sequence replacement can alter the targeting of Ig and TCR gene segments and represents an additional pathway for the generation of antibody diversity.

B. *Purification and characterization of NBP, a protein that specifically binds an enhancer of Ig gene rearrangement.* Previous work in Dr. Desiderio's laboratory identified a protein in nuclei of lymphoid cells and tissues that specifically binds DNA fragments containing Ig recombinational signal sequences. In the past year the protein's DNA recognition site has been defined precisely, the *in vivo* function of this site has been examined, and the protein has been purified to homogeneity.

To define the protein's binding site, a series of mutant substrates was generated. Mutations within the nonamer resulted in large (300- to >1,000-fold) decreases in affinity; mutations outside the nonamer had at most a 10-fold effect. Thus the protein's recognition site coincides with the conserved nonamer. The protein has therefore been called NBP, for nonamer-binding protein. If specific recognition of the nonamer element plays a role in rearrangement, deletion of the nonamer would be expected to impair rearrangement. To test this, rearrangement of wild-type or mutant recombinational substrates was assayed *in vivo*. Deletion of the nonamer element resulted in at least a 50-fold decrease in the frequency of Ig gene rearrangement, indicating that the nonamer is a profound enhancer of rearrangement. On the basis of its sequence specificity, its preferential expression in lymphoid cells, and the impairment of rearrangement upon deletion of its binding site, it seems likely that NBP functions in Ig and TCR gene rearrangement.

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By a combination of conventional and recognition site affinity chromatography, NBP has been purified to homogeneity. Greater than 20,000-fold purification was achieved, with an overall yield of 22%. The purified protein (M_r 63,000) exists as a monomer in 0.5 M NaCl. The availability of affinity-purified NBP will facilitate functional studies, not least by providing peptide sequence information that should be useful in identifying the gene for NBP.

II. Molecular Mechanisms of Signal Transduction in Lymphocyte Activation.

The programs of lymphocyte proliferation and differentiation that underlie the humoral immune response are mediated by the binding of three classes of ligand—specific antigens, cell-surface adhesion molecules, and peptide hormones (lymphokines)—to receptors on the surfaces of B and T cells. A growing body of evidence implicates protein-tyrosine phosphorylation in these processes. By analogy to well-characterized growth factor receptors, some of the transmembrane proteins implicated in lymphocyte activation may exert their effects via specific tyrosine kinases. In the growth factor receptor kinases, ligand-binding and kinase domains are covalently linked. Similar association of transmembrane proteins with cytoplasmic tyrosine kinases of the *src* type can be achieved by noncovalent interactions.

A. *Identification of novel tyrosine kinase genes.* Seven members of the *src* family have been described; of these only *lck* is preferentially expressed in lymphoid cells. The involvement of tyrosine phosphorylation in multiple pathways of signal transduction in B and T cells suggested the existence of additional, lymphoid-specific members of the *src* family. To identify novel cDNA clones encoding cytoplasmic tyrosine kinases, Dr. Desiderio and his colleagues (in collaboration with Dr. John E. Niederhuber, The Johns Hopkins Medical School) used degenerate oligonucleotide probes to screen an activated lymphocyte cDNA library. The probes were based on nucleotide sequences conserved among known cytoplasmic tyrosine kinases. In a screen of 250,000 recombinant bacteriophage, seven independent cDNA clones were obtained; by cross-hybridization and nucleotide sequence analysis, these were found to represent six different members of the *src* family. Three of the clones represent known genes: *src*, *abl*, and *lck*. Two others represent murine homologues of the genes *fyn* and *yes*. Significantly, the sixth class represents a novel *src* family member, which Dr. Desiderio and his colleagues have called *blk*, for *B lymphoid kinase*.

B. *Identification of a novel protein tyrosine kinase expressed specifically in B lymphoid cells.* A nearly complete *blk* cDNA has been obtained, and its nucleotide sequence has been determined. The cDNA contains a single long open reading frame of 499 codons, encoding a 55,000 M_r polypeptide that is closely related to but distinct from previously identified retroviral and cellular protein-tyrosine kinases. The amino acid sequence of the protein encoded by *blk* shares 63% and 61% overall sequence identity with the proteins specified by *hck* and *lck*, its closest relatives.

The pattern of expression of *blk* is unique among members of the *src* family. In immortalized cell lines, *blk* was expressed only in cells of the B lineage; no expression was seen in cells of T lymphoid, myeloid, erythroid, fibroblastoid, neuronal, or hepatocellular origin. In normal murine tissues *blk* transcripts were found only in spleen. Selective removal of B cells from the population of normal spleen cells resulted in removal of *blk* transcripts, indicating that *blk* is preferentially expressed in B lymphocytes. A survey of cell lines that represent various stages in B cell development suggests that *blk* begins to be expressed early in ontogeny, prior to expression of immunoglobulin; *blk* expression continues in mature B cells. At present, *blk* is the only known tyrosine kinase gene that is specifically expressed in B lymphoid cells. Dr. Desiderio and his colleagues propose that the product of *blk* functions in a specialized signal transduction pathway in B lymphocytes. Future experiments will address the function of *blk* and the molecular basis of its tissue specificity.

Dr. Desiderio is also Assistant Professor of Molecular Biology and Genetics at The Johns Hopkins University School of Medicine.

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TRANSCRIPTIONAL CONTROL IN EARLY *DROSOPHILA* DEVELOPMENT

CLAUDE DESPLAN, PH.D., *Assistant Investigator*

The information required for the development of a fertilized egg into a complete organism is encoded in the genetic material of the zygote provided by both parents, as well as in products loaded by the mother during the formation of the egg. The availability of genetics in the fruit fly *Drosophila* has allowed the identification of many of the genes involved in the process of development. Many genes appear to encode transcription factors and to share common protein domains. Through a network of transcriptional regulators, the zygotic genome responds to maternal organizing factors to set up the body pattern of the embryo. The goal of the current research in Dr. Desplan's laboratory is to understand the mechanisms involved in these regulatory interactions. The functions of two classes of developmental gene products that contain either a homeodomain (HD) or zinc finger DNA-binding motif are being investigated. Of particular interest are the interactions governing DNA-binding specificity of these proteins and their action in controlling transcription of their target genes. Since several genes expressed during embryogenesis in mammals share homology with the *Drosophila* developmental genes, it is likely that the mechanisms uncovered are also of significance for the development of multicellular organisms.

I. Interactions Among Gap Genes.

The first zygotic genes to be expressed in the *Drosophila* embryo are the gap genes. Their role is to read and interpret coarse positional information deposited in the egg by the mother and to refine this information by cross-regulatory interactions. Three gap genes that have been analyzed molecularly contain zinc finger motifs characteristic of many eukaryotic DNA-binding proteins. This homology suggests that they might act as regulators of transcription. A relatively simple circuit appears to control expression of one of the gap genes, *hunchback* (*hb*). Jessica Treisman has analyzed how the products of the *Krüppel* (*Kr*) and *hb* gap genes are involved in this pathway. The Kr protein produced in *Escherichia coli* is capable of binding to the sequence AAGGGGTTAA and to related DNA sequences, while the Hb protein recognizes the consensus sequence ACNCAAAAAANTA and related sites. Synthetic repeats of these consensus sequences are also recognized, showing that they are sufficient for binding. Two Kr-binding sites that could mediate the repression of the proximal promoter of the *hb* gene by *Kr* have been identified. *In vivo* this promoter drives *hb* expression in an anterior stripe, and in the absence of *Kr* the posterior border of this domain expands into the *Kr* domain. Binding sites for the Hb protein are also present upstream of both *hb* promoters. *In vivo* these sites may allow *hb* to influence its own expression. Marcia Simpson is now investigating the *in vivo* function of these binding sites for the correct patterned expression of *hb*.

The relative simplicity of the control of *hb* by a positive activator, the homeodomain-containing protein product of the maternal gene *bicoid*, and the action of a negative factor, Kr, will allow researchers to understand how interactions of these proteins with the transcriptional machinery occur in the early syncytial embryo. For this purpose, Treisman has constructed P-element vectors containing combinations of synthetic binding sites for Bcd, Kr, and Hb behind promoters not expressed (the heat-shock basal promoter) or expressed at high level in the early embryo (the Serendipity promoter). She is looking at activation and repression by these target sites in embryos and in Schneider cell culture.

II. A Single Amino Acid Determines the Specificity of Homeodomain Proteins.

Over 20 genes involved in development encode homeodomain (HD)-containing proteins. The HD includes a domain similar to the helix-turn-helix motif present in many prokaryotic DNA-binding proteins. The HD proteins can be classified on the basis of the sequence of their presumed recognition helix. By performing an extensive *in vitro* mutagenesis of several homeodomain proteins, Jessica Treisman, Pierre Gonczy, and Malini Vashishtha have been able to define a very simple rule that determines the specificity of the several classes of homeodomains. The last amino acid of the recognition helix appears to be the necessary and sufficient determinant of the specificity of a homeodomain protein. In particular, the specificity of Paired has been changed to that of another pair-rule gene product, Fushi tarazu (Ftz), by replacing a serine by a glutamine at position 9 of the recognition helix.

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Dr. Desplan and his colleagues have also changed the specificity of Paired to that of Bicoid, by replacing the same serine with a lysine. Other site-directed mutations in the helix-turn-helix motif do not further alter the specificity. A second binding activity in Paired has also been defined. This activity is independent of the recognition helix, since major disruption of this motif leaves the binding unaffected. To address the specific function of each homeodomain, Treisman is now testing the *in vivo* activity of chimeric genes carrying a modified Paired homeodomain embedded in a *ftz* gene.

III. Transcriptional Functions of the Homeodomain Proteins Fushi tarazu and Engrailed.

The question of the transcriptional role of the homeodomain-containing proteins is addressed by using knowledge of the DNA binding of HD proteins and testing their function in an *in vitro* transcription system. The goal is not to dissect any specific promoter but to analyze the mechanisms by which combinations of HD and zinc finger proteins bound to their respective sites act on the transcriptional machinery. Yoshiaki Okhuma (then an HHMI postdoctoral fellow in the laboratory) has purified to homogeneity the Engrailed and Ftz proteins from *E. coli* overexpressing cells. In collaboration with the laboratory of Dr. Robert Roeder (The Rockefeller University), he has analyzed the transcriptional role of these proteins in a fractionated *in vitro* transcription system from mammalian cells. The use of a heterologous transcription system is justified by the recent discovery that well-known transcription factors working in such systems do contain a homeodomain and also by the observation of cross-species activities of the yeast and mammalian transcription machineries. Okhuma's first observation was that En could repress transcription of basal promoters, even in the absence of strong En-binding sites. He has shown that in this case, En was acting by binding to the TATA box and competing with the TATA-box binding factor TFIID. Formation of a committed complex by preincubation of the promoter with TFIID prevented the repression by En as well as its binding to the TATA box. The *in vivo* relevance of this observation, both in cell culture and *in vivo*, is being investigated by adding a TATA box to the *engrailed* promoter that is positively regulated by En. The TATA box containing promoter of a construction negatively regulated by En *in vitro* and in cell culture is also being replaced by a different TATA box promoter and by the *engrailed* promoter that does not contain a TATA box.

Recently Okhuma has shown that the Ftz protein could activate *in vitro* transcription in a binding site–dependent manner. This activation can be prevented by En. In this case, En seems to act by competing with Ftz for binding to the HD-binding sites. When more En protein is added, a further repression appears to be mediated by the binding to the TATA box.

This analysis leads to an investigation of both the general transcription functions carried out by the HD and zinc finger proteins and the particular properties of each gene product. Attention is now focused on the role of combinations of such factors *in vitro* and *in vivo*, and insight into the control mechanisms of the coordinate expression of the developmental genes is expected.

Dr. Desplan is also Assistant Professor and A. Meyer Fellow at The Rockefeller University.

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THE MOLECULAR GENETICS OF HUMAN CANCER

ANDREW P. FEINBERG, M.D., M.P.H., *Assistant Investigator*

Dr. Feinberg is studying two aspects of the molecular genetics of human cancer: 1) early events in neoplastic transformation and 2) the role of tumor-suppressor genes in carcinogenesis.

I. Early Events in Neoplastic Transformation.

In previous work, Dr. Feinberg had discovered that widespread hypomethylation of the DNA of human cancers occurs early in multistep carcinogenesis, involving, for example, premalignant colorectal adenomas. To understand the role of DNA methylation in the earliest stages of carcinogenesis, the laboratory has developed an *in vitro* model system to capture cells after treatment with an agent that causes DNA hypomethylation, but prior to neoplastic transformation. The mouse cell line C3H/10T1/2 was chosen for these studies because of its extremely low spontaneous transformation frequency and its long latency for transformation after carcinogen exposure. Remarkably, 5-azacytidine analogues transformed the cells at a frequency of up to 10% and did not appear mutagenic. Single cells were treated at limiting dilution in microtiter well plates with 5-aza-2′-deoxycytidine (5-aza-dCyd) for 24 h, grown to subconfluence, and each microcolony was trypsinized into two fractions; one fraction was cryopreserved for later study, and the other was replated to score for eventual transformation. In this manner ancestral pretransformed cells were isolated that are indistinguishable by both morphology and growth properties from nontransformed cells but that on continued growth eventually become morphologically transformed, anchorage independent, and tumorigenic in nude mice. This is the first time that phenotypically normal cells have been captured prior to transformation. Studies of these pretransformed cells will permit examination of the earliest events in carcinogenesis and the role of DNA methylation in transformation. The laboratory is now preparing cDNA libraries from transformants and pretransformants. Two techniques—differential screening and subtraction hybridization—are being used to identify transcripts specific for commitment to transformation.

As an additional strategy to identify the role in transformation of 5-aza-dCyd, high-molecular-weight DNA was transfected from 5-aza-dCyd–induced transformants into untreated recipient cells. The laboratory has developed a novel strategy to rescue a transfected gene from a syngeneic cell line; a cosmid clone that neoplastically transforms recipient cells has been isolated from 5-aza-dCyd–treated cells. This gene is now being characterized.

II. Recessive Tumor Genes.

Dr. Feinberg previously observed loss of heterozygosity on chromosome 11 in sporadically occurring Wilms' tumors, consistent with germline deletions of 11p13 in some patients and with Knudson's model of loss of two alleles of a recessive gene in Wilms' tumorigenesis. In the first report of a putative tumor-suppressor gene in a common human malignancy, Dr. Feinberg and his co-workers found that bladder carcinomas also lose heterozygosity on 11p. Recently the laboratory has been studying two malignancies, colorectal cancer and Wilms' tumor, for potential recessive tumor genes. Colorectal cancer offers the opportunity to time genetic changes during multistep carcinogenesis, since normal mucosa, premalignant polyps, and carcinomas can be studied at progressive pathologic stages. With collaborator Gilles Thomas (Institut Curie, Paris), the laboratory observed loss of heterozygosity on chromosomes 5, 17, and 18, often within the same tumor. Losses on chromosomes 5, 17, and 18 were seen in early carcinomas, and chromosome 17 and 18 losses were also associated with tumor progression. These data suggest that altered gene dosage at multiple loci, rather than a simple Mendelian model, may be involved in multistep colorectal carcinogenesis.

It was also found that chromosome 17 and 18 losses are not independent, and they are also significantly associated with changes in ploidy. Cancers of the left side of the colon were much more likely to show allelic losses than cancers of the right side, even though the frequency of mutation at c-Ki-*ras* was constant. These data suggest a possible mechanistic association between losses on multiple chromosomes and suggest that the losses might account for the known biological differences between left- and right-sided colorectal tumors. The laboratory is now determining which molecular changes are specifically associated with defined stages of tumor progression or prognostic outcome.

The laboratory also reexamined Wilms' tumor (considered a paradigm of Knudson's model), in

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which cancer arises after loss of both alleles of a recessive tumor gene. Since children with Wilms' tumor, aniridia, genitourinary malformations, and retardation (WAGR syndrome) show overlapping deletions involving 11p13, it had been assumed that the smallest region of overlap of allelic losses in sporadic tumors would be 11p13. However, that assumption had not been tested directly. Careful mitotic mapping of chromosome 11 on 21 sporadic Wilms' tumors was performed, using 10 polymorphic markers for 11p. In five of seven tumors with allelic loss on 11p, the loss was limited to the region distal to the WAGR region. The common region of overlap of allelic losses was distal to the γ-globin gene on 11p15.5, 50 cM from the previously predicted region. Although these data do not exclude the existence of a Wilms' gene on 11p13, they raise the possibility of a second locus on 11p15.

Based on this observation, genetic linkage analysis was performed, using probes from 11p13 and 11p15, on two families with Beckwith-Wiedemann syndrome (BWS), a dominantly inherited disorder that predisposes to Wilms' and other embryonal tumors. BWS was linked to the insulin gene, on 11p15.5, with an overall maximum lod score of 3.60 (Θ = 0.00). The existence of a dominantly transmitted cancer predisposition gene on 11p15 and loss of heterozygosity for the same region in sporadic tumors provide strong evidence for a candidate-recessive tumor gene on 11p15. The laboratory has recently identified two bladder carcinomas with allelic loss also limited to 11p15. Thus loss of heterozygosity on 11p may reflect a generalized tumor progression gene at 11p15. Such a role would not necessarily contradict a role in tumor initiation as well, since the retinoblastoma gene, for example, may also be involved in some lung and breast cancers. The laboratory is attempting to localize and isolate the BWS gene, which may play a role in abnormal prenatal growth, predisposition to cancer, and tumor progression in common malignancies.

Dr. Feinberg is also Associate Professor in the Division of Medical Genetics, Department of Internal Medicine, and Assistant Professor of Human Genetics at the University of Michigan Medical School.

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PERIPHERAL TOLERANCE TO MAJOR HISTOCOMPATIBILITY COMPLEX ANTIGENS

RICHARD A. FLAVELL, PH.D., *Investigator*

Dr. Flavell's laboratory has used transgenic animals to study immune function and immune tolerance to the class II gene products of the murine major histocompatibility complex (MHC). Class II molecules of the MHC comprise two chains, called α and β, which form Ia antigens and serve as a recognition element for $CD4^+$ T cells. In the mouse, both Ia antigens, I-A and I-E, are composed of an α- and a β-chain. Strains of mice that lack I-E have served as a paradigm for studying I-E function in the mouse.

I. Peripheral Tolerance to I-E.

Considerable evidence suggests that tolerance to antigens on bone marrow–derived cells occurs primarily by deletion of reactive T cells during the development of those cells in the thymus. Mechanisms of tolerance to peripheral antigens, by contrast, have been largely obscure. If deletion of T cells is the mechanism, how then can the antigen expressed at a distal site from the thymus be translocated to the thymus in sufficient quantity to cause deletion of those reactive cells? A number of alternative hypotheses can be raised to explain tolerance to peripheral antigens. To distinguish among these, Dr. Flavell's laboratory directed the expression of the class II I-E protein to the β islet cells and the acinar cells of the pancreas, using the insulin and elastase promoters, respectively. Mice transgenic for these constructs express these genes, and hence the I-E protein, in a tissue-specific manner; the resultant mice are tolerant to the I-E antigen in both cases. In collaboration with Drs. Ralph Brinster (University of Pennsylvania) and Richard Palmiter (HHMI, University of Washington), Dr. Flavell and his co-workers have attempted to understand the mechanism of tolerance to the antigens expressed.

Tolerance does not seem to be a result of deletion of I-E–reactive T cells, since both $V\beta17a^+$ and $V\beta5^+$ T cells (both of which are deleted in control I-E+ mice) are present at normal frequency in these transgenic mice. To test whether the potentially I-E–reactive T cells in these mice can respond to antigen, Dr. Flavell and his co-workers stimulated these cells by crosslinking the T cell receptors (TCRs), using antibodies specific for I-E–reactive T cells (Vβ17a and Vβ5). This procedure induces T cell proliferation in control SJL mice but not in insulin I-E transgenic mice. T cell proliferation of these populations was not observed in several experiments, suggesting that this population is in a state of paralysis. This clonal paralysis appears to be specific for the I-E–reactive T cell population and not general immune suppression. Thus the total T cell population of the transgenic mice is responsive to crosslinking of the receptors using anti-CD3, which is present on all T cells. Crosslinking of the receptors of the Vβ8 T cell population, which is not I-E reactive, induces T cell proliferation in both transgenic and nontransgenic mice.

These initial indications suggest that tolerance to the I-E molecule expressed in the periphery is the result of clonal paralysis of T cells. Similar phenomena of clonal paralysis have been observed previously *in vitro* by several groups, notably by Dr. Ronald Schwartz and his colleagues. These data are interpreted to mean that normal T cell activation requires that the antigen MHC complex be recognized by the TCR in conjunction with a second signal to be delivered by the antigen-presenting cell. Perhaps the clonal paralysis of I-E–reactive T cells results from recognition of the I-E protein on β cells of the pancreas, which are incapable of providing that second signal. It is not clear what the defect is in paralyzed T cells, nor what that second signal is.

Tolerance in the elastase I-E mice seems to have some parallels to that described above but is not identical. As stated above, I-E–reactive cells appear not to be deleted, there is only partial clonal paralysis of the Vβ5 population, and there is little if any paralysis of the Vβ17 population. When nontolerant T cells were adoptively transferred into irradiated or T cell–depleted elastase transgenic mice, the acinar tissue was rapidly destroyed, suggesting that the I-E molecule on the acinar tissue is clearly antigenic. However, when nontolerant T lymphocytes were transferred into nonirradiated transgenic mice, this destruction did not occur. Moreover, nontolerant T cells injected into otherwise unmanipulated elastase I-E mice can be primed *in situ* to destroy the acinar tissue by injection into the same animal of I-E+ spleen cells that are themselves capable of antigen presentation. Priming the elastase transgenic mice with I-E+ spleen antigen-presenting cells does not induce an autoimmune response to the I-E+ acinar cells.

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II. Immune Function and Dysfunction in Transgenic Mice Expressing Human CD4 and HLA-DQ Genes.

This year Dr. Flavell's laboratory initiated studies that attempt to examine aspects of the functioning of the human immune system in the mouse. If these human molecules will function in a murine environment, it should be possible to study their functioning within an immune system *in vivo* and to analyze certain aspects of pathology related to the human immune system. In an initial attempt, transgenic mice expressing the human CD4 gene and the HLA-DQ 3.2 gene have been generated, in collaboration with Dr. Dimitris Kioussis (National Institute for Medical Research, Mill Hill, London, England). The human CD2 promoter/enhancer, which, unlike the CD4 promoter, has been shown to function in transgenic mice, was used to express CD4. The CD4 gene seems to be expressed in thymocytes and peripheral T cells, as well as in B cells (since it has recently been shown that murine CD2 is expressed in B cells, the latter result is expected). HLA-DQ appears to be expressed in the same sites and at about the same level as the normal murine class II genes. Current studies are directed at determining whether these human elements function in the setting of the murine immune system and in using these mice as models for acquired immune deficiency syndrome (AIDS) pathogenesis.

III. Regulation of MHC Gene Expression.

Dr. Flavell's laboratory is also studying the regulation of MHC class I and class II gene expression. Work in collaboration with the laboratory of Dr. Phillip A. Sharp (Massachusetts Institute of Technology) has shown that transcriptional activation of class I genes is explained at least in part by the binding of a DNA-binding factor (IBP-1) to an interferon response element in the promoter region of the class I gene (using in this case the *H-2K^b* gene). During this year they have shown this factor to be a molecule of ~59 kDa and further defined the DNA regions to which it binds. Current efforts are directed at cloning the gene that encodes this protein and understanding the ways in which it mediates the interferon signal.

Dr. Flavell is also Professor of Immunobiology at Yale University School of Medicine.

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HUMAN MOLECULAR GENETICS AND GENE MAPPING

UTA FRANCKE, M.D., *Investigator*

Research in Dr. Francke's laboratory has focused on mapping of genes to chromosomal sites and on molecular genetic studies of muscular dystrophy.

I. Comparative Mapping of Human and Mouse Chromosomes.

Dr. Francke's laboratory has contributed to the gene map of humans and mice by mapping a number of genes of known function to their chromosomal sites in both species. The goal of this research is a detailed knowledge of the evolutionary relationships between human and mouse chromosomes. With this information, hypotheses can be derived regarding candidate genes involved in human genetic disorders and in mouse mutations that have been described only on the phenotypic level.

The possibility was tested that the gene for adipsin, a regulatory protein that is produced in fat cells, could be involved in the genetic forms of obesity in mice (*ob* on chromosome 6 and *db* on chromosome 4). Although adipsin expression is greatly reduced in homozygous *ob* or *db* mice, the mouse adipsin locus was found to be on chromosome 10; thus it cannot be the site of the mutation in the genetically determined forms of obesity.

Inhibin (INH) is a gonadal glycoprotein hormone that regulates secretion of pituitary follicle-stimulating hormone (FSH). Two forms of inhibin that strongly inhibit FSH secretion share the same α-subunit, covalently linked to one of two distinct β-subunits (β_A or β_B). However, dimers of two β-subunits are potent stimulators of FSH synthesis and release. With cDNA probes (obtained from Dr. Anthony Mason), all three inhibin subunit genes have been mapped on human and mouse chromosomes by Southern blot analysis of somatic cell hybrid DNA and by *in situ* chromosomal hybridization. INHα and INHβ_B genes were assigned to human chromosome 2, regions q33→qter and cen→q13, respectively, and to mouse chromosome 1. The INHβ_A locus was mapped to human chromosome 7p15→p14 and mouse chromosome 13. The region of mouse chromosome 1 that carries other genes known to have homologues on human chromosome 1q includes the locus for juvenile spermatogonial depletion (*jsd*). An inhibin gene could be involved in the *jsd* mutation, since adult homozygous male mice have elevated levels of serum FSH and their testes are devoid of spermatogonial cells. When the INHα and INHβ genes in *jsd* mice were examined by Southern blot analysis, no evidence for major deletions or rearrangements was detected. The possibility that a point mutation in an inhibin gene is responsible for the *jsd* phenotype is being investigated.

Receptor genes mapped in previous years in the human have now been assigned to mouse chromosomes, in all cases to regions of known conserved synteny. The insulin-like growth factor I receptor gene is on mouse chromosome 7 concordant with the c-*Fes* proto-oncogene. In the human both loci are very close to each other, on the distal long arm of chromosome 15. Two receptor genes that are close to each other on human 5q were found to be syntenic on mouse chromosome 18. These are the genes for the β_2-adrenergic receptor and the platelet-derived growth factor (PDGF) receptor. The glucocorticoid receptor gene (*GRL*) had previously been assigned to human chromosome 5 and mouse chromosome 18 by Dr. Francke and her colleagues. Regional mapping data reported by others had placed *GRL* on the proximal part of 5q, which is a region that contains loci homologous to genes on mouse chromosome 13, not 18. Therefore *in situ* hybridization studies were carried out with a human *GRL* probe. Results reassigned the *GRL* gene to 5q31→5q32, consistent with expectations. This new location of GRL also suggests a potential relevance of its loss in 5q– deletions that are seen in hematopoietic disorders.

II. Chromosomal Mapping of Human Genes Potentially Involved in Genetic Disorders.

Efforts to map human genes to sites on human chromosomes have focused on 1) cloned genes known to be involved in genetic diseases; 2) genes specifically expressed in muscle or the nervous system that could potentially be involved in inherited disorders; and 3) genes involved in growth control, such as growth factors, their receptors, and other cell surface antigens or second messengers. The rationale for the last is to see whether these genes could be colocalized with one of the 70 sites known on human chromosomes that show preferential breakage in malignant cells. These studies were done in collaboration with the laboratories that had cloned the genes (see PUBLICATIONS).

Blast-1 is a human activation-associated glycoprotein expressed on the surface of leukocytes. The lo-

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cation of the Blast-1 gene on chromosome 1 bands q21–q23 puts it near the CD1 cluster of immunoglobulin superfamily genes, suggesting that they may be evolutionarily related. The gene for a trifunctional protein that catalyzes three consecutive steps in the interconversion of tetrahydrofolate derivatives was mapped to human chromosome 14q24. A second, more weakly hybridizing sequence was found on the short arm of the X chromosome. The function of this sequence is not known. The site on chromosome 14 is the expressed gene, consistent with previous studies of the expression of the enzyme in somatic cell hybrids.

Cytochrome *c* oxidase (COX) is the terminal enzyme of the respiratory chain and consists of at least 13 subunits, three of which are mitochondrial encoded and have catalytic activity. In addition, 10 smaller nuclear-encoded subunits exist that show tissue-specific expression. Systematic chromosomal mapping of the nuclear subunit genes is being undertaken as cloned probes become available. So far, most of the cDNAs for small subunits hybridized to more than one chromosomal site, and the identification of the coding sequence has been difficult. Subunit VIII, however, was localized to a single site on the proximal long arm of chromosome 11, even though there are different isoforms in muscle and nonmuscle tissue. COX deficiency is associated with a variety of human encephalomyopathies. The cloning and mapping of nuclear COX genes should lead to a genetic dissection of this heterogenous group of disorders.

Phosphorylase kinase (PHK), the enzyme that activates glycogen phosphorylases in muscle, liver, and other tissues, is composed of four different subunits. The largest subunits, α and β, have been cloned recently from rabbit muscle cDNA. Dr. Francke's laboratory has used these probes to map the gene in humans by rodent x human somatic cell hybrid panels as well as *in situ* chromosomal hybridization. Both genes were found to exist at single sites. The α-subunit gene is located on the proximal long arm of the X chromosome in region Xq12–q13 near the locus for phosphoglycerate kinase (*PGK1*). X-linked mutations leading to PHK deficiency, known to exist in humans and mice, are likely to involve this locus. The PHK deficiency mutation *Phk* has been mapped near *Pgk-1* on the mouse X chromosome. The β-subunit gene *PHKB* was mapped to chromosome 16 region q12–q13. This information should help when families with autosomally inherited PHK deficiency are studied for mutation at this locus.

To facilitate the correlation of the physical chromosome map with the genetic linkage map, searches for restriction fragment length polymorphisms (RFLPs) are carried out with clinically relevant probes. A common *Sac*I polymorphism was found in the gene for the M1 subunit of ribonucleotide reductase that was previously assigned to the distal short arm of chromosome 11. The gene encoding the X-linked form of chronic granulomatous disease was found to be polymorphic with *Nsi*I. This RFLP should be useful for linkage mapping of region Xp21.1.

A human endogenous retroviral element related to the simian sarcoma-associated virus was used for chromosome mapping studies. One molecular clone was found to hybridize predominantly to a site on human chromosome 18q21 and to recognize two RFLPs. This probe adds a useful marker to the linkage map of chromosome 18.

III. Molecular Genetic Studies of Muscular Dystrophy.

Since dystrophin cDNAs and dystrophin-specific antibodies have been made available by Dr. Louis M. Kunkel (HHMI, Harvard Medical School) and his colleagues, the presence and expression of this gene has been studied in individuals affected with early onset progressive muscular dystrophy. Dr. Francke's laboratory previously published the characterization of partial deletions of this gene, the origin of the mutations, and the discovery of germline mosaics in both males and females. Recent research has focused on the identification of families with a clinical disorder similar to Duchenne muscular dystrophy (DMD), in which the dystrophin gene is structurally normal and the dystrophin protein is expressed in normal size and abundance. Such families came to the attention through involvement of an affected girl with a normal chromosome constitution. Genetic marker studies using RFLPs from the region containing the DMD gene on Xp have provided conclusive evidence that in two families this gene was not involved. In a third family the manifesting female was a heterozygote for the same partial DMD gene deletion present in her affected brother. In this case, nonrandom X inactivation can explain the findings. The existence of an autosomal recessive form of early onset progressive muscular dystrophy has previously been suggested. Recently a cDNA that is partially homologous to the 3′ end of the dystrophin sequence has been found to map to human chromosome 6. This is now being tested

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as a candidate gene in these families. The elucidation of the nature of the autosomal mutations may lead to a better understanding of dystrophin regulation, function, and interaction with other muscle proteins.

Dr. Francke is also Professor of Genetics and of Pediatrics at Stanford University School of Medicine. Since Dr. Francke joined the Institute in January 1989, much of the work reported was also supported through the National Institutes of Health.

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BRAIN PEPTIDES AND FEEDING BEHAVIOR

JEFFREY M. FRIEDMAN, M.D., PH.D., *Assistant Investigator*

Numerous experiments in humans and in other mammals have suggested that feeding behavior and body weight are tightly regulated. These studies 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 states that each individual has a "set point" that determines how much he or she 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 level of peripherally synthesized molecules (hormones perhaps) reflects the nutritional state of an individual and that the levels of these "satiety factors" are sensed by feeding control centers in the hypothalamus and elsewhere. The identity of these satiety factors, however, remains to be elucidated. Dr. Friedman's laboratory is taking two approaches to the elucidation of factors involved in the control of feeding behavior. The first approach is aimed at the molecular cloning of two recessive mouse mutations, obese (*ob*) and diabetic (*db*), that result in profound obesity. The obesity in these animals is the result of abnormalities in feeding behavior and energy expenditure and may reflect a genetic defect in the set point. The second approach involves a detailed molecular analysis of the regulation and function of a candidate satiety factor, the brain-gut peptide cholecystokinin (CCK).

I. 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 a second mutation, diabetic (*db*), was identified by Dr. Douglas Coleman; when bred on the same genetic background this mutation has a phenotype identical to *ob*. This mutation was genetically localized to mouse chromosome 4. 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 were missing a circulating factor that suppresses appetite and that animals with the *db* mutation were missing a receptor for this satiety factor. It has proven difficult, however, to identify the *ob* and *db* gene products directly, because the profound obesity present in these animals results in numerous secondary endocrine and biochemical abnormalities. The primary defect in these animals does not appear to be an abnormality in CCK metabolism, since Dr. Friedman and his colleagues have shown, in collaboration with Dr. Uta Francke (HHMI, Stanford University), that this gene is present on mouse chromosome 9. Furthermore, Dr. Friedman's group has not found a difference in the levels of CCK in the brain and intestine of obese and non-obese animals.

The technology of reverse genetics is being used to clone the *ob* and *db* genes (in collaboration with Dr. Rudolph L. Leibel). The first step in this strategy involves the localization of the mutant gene to specific regions of the genome, using restriction fragment length polymorphisms (RFLPs).

A standard backcross of 500 animals (to date) between *ob/ob* C57BL/6J mice and DBA mice has been generated. Polymorphic markers (RFLPs) can be easily identified among the progeny of this cross and tracked relative to the obese phenotype. One marker present on chromosome 6, *met*, has been shown to map to within 3 centimorgans (cM) of the *ob* trait. Additional polymorphic markers present on proximal chromosome 6, including carboxypeptidase A (*cpa*), *gr1*, and procollagen have been mapped among the backcrossed animals to generate a molecular map of this mutation. These experiments have demonstrated that *cpa* and *met* flank the *ob* gene and are ~5 cM apart. A similar cross has also been established for the *db* mutation. Dr. Friedman and his colleagues have mapped *db* to a position 3 cM distal to the interferon-α gene, 2 cM distal to c-*jun*, and 5 cM proximal to the liver glucose transporter gene. To be most effective, this approach requires the genetic mapping of numerous clones so as to saturate the regions in the vicinity of *ob* and *db*. To generate a bank of clones specific for mouse chromosomes 4 and 6, Dr. Friedman derived a cell line from a mouse that carried a Robertsonian 4:6 translocation. Robertsonian translocations are centromeric fusions of two chromosomes. This cell line is useful because the Robertsonian chromo-

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some, by virtue of its large size, can be separated easily with a fluorescence-activated chromosome sorter. In collaboration with Scott Cram and Kevin Albright at the Los Alamos National Laboratory, Dr. Friedman's laboratory has constructed a genomic library from the flow-sorted 4,6 chromosome and is currently mapping phage clones from this resource. In addition, with the help of Dr. Steve Brown in England, Dr. Friedman's group is using microdissection and microcloning techniques to make libraries from the distal region of mouse chromosome 4 (where *db* is located) and the proximal region of mouse 6 (where *ob* is located). The use of chromosome-specific libraries of these probes for "saturation" mapping of these chromosomes is a first step toward the cloning of the *ob* and *db* genes. The presence of markers tightly linked to *ob* (*met* and *cpa*) and *db* (interferon-α and c-*jun*) should make it possible to generate physical maps (long-range restriction maps) of these regions of the mouse genome using pulsed-field gel electrophoresis. These experiments are under way and should accelerate efforts to obtain other clones that are tightly linked to *ob* and *db*.

II. Cholecystokinin Regulation and Function.

The hormone CCK was originally found in the small intestine by virtue of its ability to stimulate gallbladder contraction and pancreatic secretion in response to feeding. High levels of CCK were recently found in neurons of the mammalian brain, where it functions as a neurotransmitter. In the brain this neuropeptide is found in subsets of neurons in several regions that differ in their morphologic, functional, and biochemical characteristics.

Dr. Friedman's objective is to elucidate the molecular mechanisms controlling the expression of the CCK gene in each of the cell types that express CCK RNA. A full understanding of the regulation of this gene in each of the expressing cell types would yield insight into the genetic strategies employed in instances where the same gene is expressed in different cell types. Dr. Friedman and his colleagues are also employing a variety of techniques in molecular biology to explore the function(s) of this hormone.

Previously the laboratory cloned and sequenced the single-copy mouse CCK gene. Before the DNA sequences involved in the transcriptional control of the CCK gene could be defined, it was necessary to identify cultured cell lines that express this gene. Two separate pediatric tumor/cell lines that express the CCK gene were initially identified. Both tumors were derived from neuroepitheliomas, which are primitive nerve tumors that usually develop in the chest wall. Dr. Maria Vitale has used these cell lines to demonstrate that cell-specific expression of the CCK gene in cultured neuroepithelioma cells and cell-specific repression in cultured cells that do not synthesize CCK mRNA require the presence of DNA sequences between −6.5 and −13.5 kb. A more precise localization of these DNA sequences is currently being determined by deletion analysis.

After observing that CCK RNA is present in two neuroepitheliomas, Dr. Friedman screened eight additional tumor cell lines of this type and discovered that all neuroepitheliomas synthesize this mRNA. Eight out of eight cell lines derived from a pediatric bone tumor thought to resemble neuroepitheliomas, Ewing's sarcoma of bone, also express CCK mRNA. Subsets of other pediatric tumors, including rhabdomyosarcoma, a malignant muscle tumor, also appear to express the CCK gene. Since CCK is a neural marker, these data suggest that there may be a class of pediatric nerve cell tumors that express this hormone and that synthesis of this peptide may be of diagnostic value in pediatric solid tumors. The possibility that CCK-producing tumors have a different clinical course and require different therapy than tumors that do not synthesize this hormone needs to be considered. Most of these tumors do not, however, process the CCK prohormone and hence do not synthesize immunoreactive CCK. They do secrete the CCK protein precursor, which can be detected by use of a novel radioimmunoassay to the carboxyl terminus of the CCK precursor that was developed by Dr. Bruce Schneider and Dr. Friedman. In preliminary experiments with this radioimmunoassay, it appears that the blood levels of the CCK precursor are elevated in patients with the aforementioned tumors. These observations also suggest that ectopic CCK production could in some cases have pathophysiologic effects in humans.

To describe the implications of ectopic CCK production and to consider the role of CCK in the control of feeding behavior, Dr. Friedman has, in collaboration with Drs. Richard Palmiter (HHMI, University of Washington) and Ralph Brinster (University of Pennsylvania), artificially expressed high levels of this hormone in blood by fusing the metallothionein (MT) promoter to the CCK coding sequence and introducing the MT-CCK transgene into transgenic mice. Transgenic mice

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that express high levels of CCK in liver, plasma, and elsewhere are now available and are being characterized.

Dr. Jeffrey M. Friedman is also Assistant Professor in the Laboratory of Molecular Cell Biology at The Rockefeller University.

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GENETIC RECOMBINATION IN MURINE GERM CELLS

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).

Histocompatibility molecules are the proteins found on the cells of mice and humans that are responsible for transplant rejection. The biological function of these molecules is to bind viral, bacterial, and tumor antigens and present them to the immune system. The immune system can then attack and kill the infected cells and stop the spread of infection. Individual mice and humans have from three to six different histocompatibility molecules, each of which can bind a limited number of antigens. To bind a large number of these antigens and ensure the survival of the species, it is beneficial that many varieties of the histocompatibility molecules be found 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, which also includes the *Qa* and *Tla* region genes. Alleles of each *H-2* locus exhibit high-sequence variation (diversity) and are very polymorphic at the population level. 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.

Mutant K^b histocompatibility genes have been detected in C57BL/6 mice at the high frequency of ~1 per 2,500 (alleles of *H-2* loci from the C57BL/6 strain are identified by a *b* superscript). Because they are histoincompatible with other members of the same inbred mouse strain, mice with mutant *H-2* genes have been detected by skin grafting. Sequence analyses have indicated that these mutant K^b genes contain clustered, multiple nucleotide alterations. The mutant K^b genes contain from two to seven nucleotide substitutions compared with the parental gene. Using oligonucleotide probes that are complementary to the mutant sequence in the altered K^b gene, Dr. Geliebter has determined that the mutant K^b genes are generated by recombination between the K^b gene and other class I genes. The mutant-specific oligonucleotide probes were hybridized to clones containing all of the class I genes of the C57BL/6 mouse, and upon sequencing, the positive clones were found to contain the identical sequences as substituted into the mutant K^b genes.

These studies provided evidence that mutant histocompatibility genes arise via recombination events between *K* genes and other class I donor genes and result in the transfer of very small segments of DNA (<100 nucleotides) from donor genes to the K^b gene. These very short genetic transfers have been termed *microrecombinations*. The microrecombinant K^b genes are repetitive in nature; that is, the identical microrecombinant K^b gene has been detected in independently arising mutant mice. It is thought that the products of many microrecombination events, accumulating in the *K* genes of a mouse population, result in the high-sequence diversity observed among *K* alleles.

Although it is certain that microrecombinations occur in germ cells of normal C57BL/6 mice, little is known about the molecular parameters of the microrecombination process. For example, it is not known whether microrecombinations proceed via a reciprocal (double crossover) or a nonreciprocal (gene conversion) mechanism. Furthermore, there is some genealogical evidence that microrecombination occurs only in female mice. In addition, microrecombinations may not occur in all strains of mice or with equal frequency in all *H-2* genes of the same mouse. Further analyses of these aspects of the microrecombination process using classical skin graft techniques is either impractical or impossible.

The emphasis of the research in Dr. Geliebter's laboratory is the analysis of the parameters and requirements of the microrecombination process. The approach being taken is the analysis of microrecombination products in murine germ cells; since microrecombinant mice come from microrecombinant germ cells found in normal parents, the germ cells of normal mice should contain many microrecombinant K^b genes (~1 in 5,000). In fact, since ovarian tissue from a single C57BL/6 fetus contains 25,000 germ cells (100,000 K^b genes) there should be ~20 microrecombinant K^b genes per normal mouse. Since microrecombination events have been found to reoccur in nature, oligonucleotide probes for known microrecombinant

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genes can be used to detect repetitive, independent mutants containing the same sequence.

DNA from C57BL/6 ovaries is being prepared, followed by the amplification and cloning of all of the K^b genes, both wild type and microrecombinant. Screening the K^b clones for one or several particular microrecombinants is accomplished with a single oligonucleotide probe or a cocktail of oligonucleotide probes for known microrecombinant genes.

The amplification of K^b genes is accomplished using K^b-specific oligonucleotide primers and the polymerase chain reaction (PCR). Using a series of PCR reactions, Dr. Geliebter's laboratory has been able to achieve a 10- to 100-million-fold amplification of the K^b gene. The inclusion of restriction enzyme sites on the oligonucleotide primers facilitates the cloning of the amplified product, to the extent that K^b libraries with over 1 million clones are achievable. An elaborate scheme of gel purifications of the K^b gene, both before and after the PCR reactions, has been implemented, to avoid possible artifacts inherent in the PCR.

This amplification and cloning scheme should allow for the accurate determination of microrecombination frequencies of *H-2* genes. These data will enable the comparison of microrecombinations in females versus males, different strains of mice, and different *H-2* genes within the same mouse strain. Furthermore, the construction of donor gene libraries by the same procedures, followed by screening for K^b sequences, will determine if *Qa* and *Tla* region genes are recipients of *H-2* DNA sequences. This will help determine if the microrecombination process is reciprocal or nonreciprocal.

Dr. Geliebter is also Assistant Professor and University Fellow at The Rockefeller University.

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GENETIC ANALYSIS OF GROWTH CONTROL

DONNA L. GEORGE, PH.D., *Assistant Investigator*

Cellular proliferation and differentiation are intricately regulated; both are controlled by the coordinated interactions of a large number of genes. Genetic events that lead to the altered or inappropriate expression of any of a broadly based class of genes, included among the proto-oncogenes, can result in the perturbation of normal cell growth and development; such uncontrolled proliferation and concomitant loss of differentiative ability are hallmarks of transformation and tumorigenesis. Research in this laboratory has focused on the identification and characterization of such growth-control genes, as well as the molecular mechanisms governing their expression.

I. Amplified DNA Sequences in a Transformed Mouse Cell Line.

A number of studies have demonstrated that the amplification and consequent overexpression of particular cellular genes play a direct role in the etiology of some tumors. This laboratory is currently characterizing the function and organization of amplified DNA sequences located in stably maintained extrachromosomal double minute nuclear elements (DMs) in a spontaneously transformed derivative of mouse 3T3 cells (3T3-DM).

No known oncogene is amplified in these cells. However, because of the stable presence of DMs in these cells in the absence of selective pressure, the amplified domain is expected to contain a previously unrecognized gene with a role in cellular proliferation/growth control. A multifaceted approach has been used for the analysis of the amplified DNA, including differential screening of cDNA libraries, chromosome walking, cDNA and genomic cloning, and pulsed-field gel mapping of CpG island-associated transcription units. It would appear that three genes are amplified and overexpressed in these cells. To date there is no evidence for the presence or overexpression of other amplified genes.

For each of these three amplified genes, a full-length genomic equivalent has been introduced into nontransformed recipient cells and amplified appropriately to mimic the state in 3T3-DM cells. Results obtained in repeat studies indicate that the overexpression of one of these genes (*mdm-1*) in Rat-2 cells is associated with a distinct morphological alteration that is characteristic of some transformed derivatives. Additional experiments, including cotransfection studies with c-*myc* and nude mouse injections, are in progress to confirm and extend these findings. Because of the potential role of this gene in a pathway of cellular growth control, it is of interest that immunofluorescence studies have provided evidence that at least one of the *mdm-1*–encoded proteins is nuclear localized, in a pattern representative of a nuclear matrix component; in addition, this gene has enhanced expression developmentally during spermatogenesis.

An intriguing observation has been made concerning another of the amplified genes in the 3T3-DM cells. A cDNA probe from this gene detects an mRNA species that is present in several normal tissues and nontransformed cell lines but is conspicuously absent in a number of tumorigenic cell lines. If these initial studies are substantiated, Dr. George and her colleagues will proceed with studies to address how this differential expression might be related to the transformed properties of these tumors.

II. Promoter Region of the cKi-*ras* Proto-oncogene.

Another area of investigation is aimed at elucidating sequence elements mediating transcription of the cKi-*ras* proto-oncogene. Alterations in the structure or level of expression of this gene have been implicated in the etiology of a number of mammalian tumors. Enhanced expression of an altered cKi-*ras* gene has been shown to augment the metastatic potential of some primary tumor cells. Thus it is important to clarify the various factors that regulate the expression of this gene.

Results obtained in transient expression assays allowed the identification of a 160 bp DNA fragment critical for promoter activity. Subsequently, gel mobility shift and DNase protection assays indicated that this region is composed of at least two sequence domains that bind nuclear proteins and most likely represent transcriptional regulatory elements. One domain includes a binding site for the transcription factor Sp1. Another domain, highly conserved among human and mouse cKi-*ras* genes, shows no sequence homology to binding sites for previously identified transcription factors and may represent recognition elements for as yet uncharacterized DNA-binding proteins. Included in this latter domain is a homopurine-homopyrimi-

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dine motif found in the promoter regions of a number of eukaryotic "housekeeping" genes, such as c-*myc* and genes encoding the insulin and epidermal growth factor (EGF) receptors.

Utilizing Southwestern screening of an expression library, Dr. George and her colleagues have identified cDNA clones encoding two of these putative binding proteins, including one that binds to the homopurine-homopyrimidine domain. Because the cKi-*ras* promoter region contains features shared by other housekeeping genes, efforts to understand how transcription of this gene is governed should reveal factors regulating the expression of a class or set of such growth-control genes.

Dr. George is also Associate Professor of Human Genetics at the University of Pennsylvania School of Medicine.

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GENE EXPRESSION AND ORGANIZATION

RAYMOND F. GESTELAND, PH.D, *Investigator*

I. Unusual Translation Events.

A. *Frameshifting and hopping* (Dr. John F. Atkins, Michael O'Connor, Therese Tuohy, and Norma Wills). Michael O'Connor completed work on mutants of $tRNA^{Val}$ that enhance short-distance hopping from one valine codon to another. One class of mutants has a single-base insertion in its anticodons. The hopping efficiency of these mutants dropped sharply with increasing distance. Low-efficiency hopping occurs over three tandem stop codons, whereas in certain mRNA contexts, hopping between two valine codons spaced two nucleotides apart occurs with 20% efficiency. The other class of mutants has a base substitution in the third nucleotide from the 3′ end of the tRNA, changing CCA to GCA or ACA. Norma Wills, who is investigating the tRNA from these mutants, found (in collaboration with Dr. Lionello Bossi) an equivalent change in a mutant $tRNA^{His}$ that altered the regulation of the histidine biosynthetic operon. This class of mutants may provide information about the interaction between the 3′ end of tRNA and the peptidyl transferase region of ribosomal RNA.

B. *Large hops* (Dr. Robert Weiss and Diane Dunn). The laboratory is continuing to examine the hopping of ribosomes over a 50 nucleotide (nt) interruption in the mRNA of bacteriophage T4 gene *60* with nearly 100% efficiency. A mutational analysis of the mRNA elements required has unexpectedly revealed that the peptide sequence encoded in the 138 nt 5′ of the jump site is required for high-level bypass of the interruption, as if the growing polypeptide chain itself is somehow promoting this event. Mutations that disrupt the interrupt also indicate that the gene *60* jump is analogous to tRNA hopping, previously observed at low levels in *Escherichia coli*. Also it is apparent that appropriate secondary structure is needed at certain sequences of the interrupt.

C. *Retroviral frameshifts* (Dr. R. Weiss and D. Dunn). With most retroviruses the coupling of *gag-pol* expression is effected by ribosomal frameshifting; the sequences required include certain tandem codons plus some downstream sequence that at least in one case involves a pseudoknot structure. Expression of retroviral ribosomal frameshift sites in *E. coli* as retroviral *gag-pol/lacZ* fusions has shown that these prokaryotic ribosomes can respond to the retroviral mRNA signals that provoke high-level ribosomal frameshifting. This has provided a convenient system in which to analyze the components of these frameshift sites comprising a special slippery tRNA context and a 3′-flanking mRNA element.

D. *Plant viruses* (Dr. James Skuzeski and Linda Nichols). Several plant RNA viruses encode read-through proteins, where suppressor tRNA reading of a "leaky" stop codon has been implicated. However, in three unrelated viruses, the sequence around the read-through site is conserved (CAA-TAG-CAA-TTA), with an amber stop codon flanked by two glutamine codons. The recent demonstration that tRNAs can hop over stops flanked by homologous codons in *E. coli* suggests an alternative explanation. Hopping is being tested by transfection of tobacco protoplasts with constructs containing a β-glucuronidase reporter gene. Identity of the 3′ CAA codon is crucial as predicted if hopping were involved. Surprisingly, however, read-through levels are also drastically lowered by several mutations in the UUA leucine codon, and it is unclear how this fits with either the suppression model or hopping. Protein sequence information is crucial. Stably transformed tobacco cell lines that express the gene have been established for this purpose.

E. *T7 frameshifting* (Barry Condron). The major capsid gene of bacteriophage T7 has a frameshift product. Experiments with fusions suggest that sequences far removed on the mRNA may be involved.

F. *Messenger RNA stability* (Lori Wagner and Dr. Weiss). Sequence elements near the 5′ end of mRNAs can affect mRNA stability. It is unknown if these act by determining nuclease sensitivity directly or by altering ribosome initiation frequency. A single-base change in the fourth codon of *lacZ* decreases mRNA stability threefold. The effects of translation initiation on mRNA stability are being tested by inserting oligonucleotides into the *lacZ* gene, which alters the wild-type Shine-Dalgarno (SD) sequence and the initiation codon. The less efficient SDs correlate with message instability. The results may be explained by a 30 S subunit binding the SD region and protecting a ribonuclease-sensitive region.

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G. *RF2 gene of* E. coli (Liming Young). Overexpression of RF2 (ribosome release factor) inhibited the growth of *E. coli*. Deletion of the carboxyl terminus (~70 aa) inactivated RF2 activity and allowed normal growth when overexpressed. Further genetic experiments are aimed at defining the crucial region. RF2 protein has five leucine repeats in its amino terminus. Whether it is a leucine-zipper repeat requires further research.

II. Genome Structure.

A. *Bends in DNA* (Dr. Debra Milton and Mark Casper). Saturation mutagenesis of a 60 bp bend region of a naturally occurring SV40 DNA fragment has identified bases that influence the DNA bend. As expected from current models, interruption of an A tract or an alteration in the A tract phasing decreased the bend. However, G tracts influence a DNA bend when spaced at the half-period between periodically phased A tracts. Also, ApG or GpA dinucleotides are influential. Clearly, base pairs other than dA-dT are involved in influencing the DNA bend and provide a good database for further DNA model building.

B. *Organization of maize mitochondrial genome* (Dr. Christiane Fauron and Marie Havlik). In maize, the physical map of the normal mitochondrial DNA (N) has a sequence complexity of 570 kb, compared with 540 kb for the cytoplasmic male sterile type Texas (cmsT). For both genomes the entire sequence complexity can be represented on a master chromosome or as a multipartite structure by recombination at repeated sequences. A more detailed comparison of various N and cmsT mitochondrial genomes revealed some rare microheterogeneity (e.g., point mutations, small deletions/additions creating different restriction sites) between some N cytoplasms. The mitochondrial genome from two fertile revertants, V3 and V18, derived from a cmsT callus tissue culture has been determined. The sequence can be mapped onto circular "master chromosomes" of 705 kb and 818 kb, which include one large duplication and one small deletion. As found for the maize N type and cmsT mitochondrial genomes, the V3 and V18 master chromosomes also exist in a multipartite structure generated by recombination through repeated sequences.

III. DNA Sequencing Technology.

A. *Large-scale sequencing* (Dr. Weiss and D. Dunn). Attempts to sequence cosmid DNA efficiently are continuing. Two cosmids containing human genomic DNA from the neurofibromatosis (type 1) region of chromosome 17 have been randomly subcloned and sequenced using a multiplex protocol. This experiment allowed rapid accumulation of 85% of the sequence in a random fashion. Analysis of the pace of this particular multiplex strategy has impelled the implementation of a new subcloning strategy employing multiplexed self-replicating transposons. This protocol will simplify and accelerate the rapid accumulation of sequence data.

As part of this laboratory's participation in the Human Genome Project, several other avenues for improving DNA sequencing technology are being pursued. An optics laboratory for luminescent DNA sequencing has been assembled by Jeff Ives and Dr. Achim Karger to assess fluorescent and chemiluminescent substrates to probe DNA sequences on polymer membranes. The membranes are then imaged on a cryogenically cooled charge-coupled device (CCD) array. The possibility of DNA fractionation for sequencing using microbore (~74 μm) capillaries is being tested by Harold Swerdlow. Heat dissipation and power requirements are reduced substantially in capillaries, which permits gels to be electrophoresed at voltages 10 to 15 times higher than for conventional separations, thus increasing resolving power. Mike Murdock is progressing on development of an autoradiogram reader, using an artificial neural network that is trained on 5,000 sample images to iteratively evolve a set of network weights that are then used to read arbitrary gel images.

Dr. Gesteland is also Professor of Human Genetics and of Biology at the University of Utah School of Medicine.

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MOLECULAR BIOLOGY OF BLOOD COAGULATION

DAVID GINSBURG, M.D., *Assistant Investigator*

The research program of this laboratory focuses on the biology of the blood coagulation system and the molecular genetics of human hematologic diseases.

I. von Willebrand Factor and von Willebrand's Disease.

von Willebrand factor (vWF) is a plasma adhesive 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 VIIIC (the antihemophilic factor). von Willebrand's disease (vWD) is the most common inherited bleeding disorder in humans, with prevalence estimated to be as high as 1–3% of the population. This laboratory is interested in the molecular basis of vWD and structure-function analysis of the vWF protein.

The study of vWD at the molecular level has been difficult because the vWF gene is unusually large (>175 kb, >50 introns), a nonprocessed pseudogene is present, and there is no ready source of vWF mRNA from patients. In addition, more than 20 distinct subtypes of vWD have been reported, all with subtle phenotypic differences. This laboratory has recently adapted the polymerase chain reaction (PCR) to study trace amounts of vWF mRNA present in peripheral blood platelets. With this approach, two different single-base substitutions were identified in two type IIA vWD patients, both resulting in nonconservative amino acid substitutions. Both substitutions are located immediately adjacent to an important new functional domain of vWF involved in platelet binding, independently identified in the laboratory (see below). When introduced into full-length vWF cDNA and expressed by transfection into COS cells, one of these mutations resulted in loss of the large vWF multimers, similar to the pattern observed in type IIA vWD plasma. In the other case, a normal multimer pattern was seen. The major defect in type IIA vWD may be functional, due to the disruption of this novel platelet-binding domain, with loss of multimers a secondary phenomenon. Other single-base substitutions, all clustered in the same region, have recently been identified in three additional type IIA patients. In two of these unrelated families the identical base substitution was observed. Based on sequence differences upstream of the substitution, the same mutation seems to occur independently in these two families. Five potential mutations have now been identified in nine type IIA families. The effects of these substitutions on vWF function and multimer assembly are currently being investigated.

The laboratory has continued to study structure-function relationships within the vWF molecule. vWF is an unusually large and complex adhesive glycoprotein that interacts with platelets, the subendothelium, and several other clotting proteins, via a number of distinct functional domains. In collaboration with Dr. Paula Bockenstedt (University of Michigan), this laboratory has used a recombinant approach to localize specific immune epitopes precisely within vWF, for a large panel of monoclonal antibodies (MAbs). To date, 12 MAbs have been localized. Several of these identify identical epitopes, suggesting that there may be a limited number of strongly immunogenic regions within this large molecule. Two of these regions are of particular functional interest. In collaboration with Dr. David Fass (Mayo Clinic), MAbs observed to block the binding of factor VIIIC to vWF were localized to a 19–amino acid epitope near the vWF amino terminus. Further studies are focusing on this region to define more precisely the structural requirements for the important vWF–factor VIIIC binding interaction.

Another MAb observed to block vWF binding to platelets via the glycoprotein GPIb receptor was localized to a region near the midportion of vWF. When a vWF segment from this region was expressed in *Escherichia coli*, a marked effect on vWF-platelet binding was observed in an *in vitro* assay. This novel region is distinct from any previously identified vWF functional domains and may be associated with the defect in type IIA vWD (see above). These findings could have important implications for the therapy of both vWD and hemophilia A and might lead to the development of important new anticoagulant drugs.

II. Plasminogen Activator Inhibitor-1.

The fibrinolytic system is regulated, in part, by a balance between tissue-type plasminogen activator (tPA) and its specific, rapidly acting inhibitor, plasminogen activator inhibitor-1 (PAI-1). Abnormal plasma levels of PAI-1 may be associated with a variety of thromboembolic disorders in humans. Full-length cDNA clones for human PAI-1 have pre-

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viously been isolated in this laboratory, and the gene has been localized to human chromosome 7. PAI-1 was shown to be a member of the serine protease inhibitor (SERPIN) supergene family. The laboratory has now characterized the structure of the PAI-1 cellular gene that spans 12 kb and is interrupted by 8 introns. The intron-exon structure shows little similarity to that of other SERPINs, an observation with implications for the process of intron evolution. The promoter of the human PAI-1 gene has also been characterized, and its function has been demonstrated by transfection into heterologous cells. The laboratory is continuing to study the regulation of PAI-1 expression in endothelial cells.

Current studies of PAI-1 are centered largely on structure-function analysis. A system has been developed in the laboratory for expressing PAI-1 in *E. coli* as a fusion to *Staphylococcus* protein A, with a synthetic collagen sequence inserted between the *Staphylococcus* protein A and PAI-1. With this system, large quantities of PAI-1 (and several other eukaryotic proteins) have been expressed and purified, with subsequent release by cleavage with the specific protease, collagenase. This recombinant PAI-1 is functionally active and will provide the tools for future mutagenesis studies.

III. Biology of Bone Marrow Transplantation.

Bone marrow transplantation (BMT) is finding increasing application as a treatment modality for human leukemias and other malignancies. This laboratory has continued its interest in the application of restriction fragment length polymorphism (RFLP) analysis to distinguish the host-versus-donor origin of patient blood cells after BMT. The laboratory has now applied PCR to detect DNA sequence polymorphisms and is using this approach to investigate the kinetics of marrow engraftment during the first few weeks after BMT. This laboratory is also using PCR to detect chronic myelogenous leukemia cells specifically and monitor their disappearance after BMT for this disorder. These observations may have important implications for transplantation biology and for the continued development of BMT as a treatment modality.

Dr. Ginsburg is also Assistant Professor of Internal Medicine and of Human Genetics at the University of Michigan Medical School.

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ANALYSIS OF GENES IN HUMAN Xq28

JANE GITSCHIER, PH.D., *Assistant Investigator*

Mutations in X-linked genes account for ~15% of inherited disease in humans. A number of these loci genetically map to Xq28, a region of DNA flanked by the long-arm telomere and the fragile site associated with mental retardation. This laboratory is pursuing several lines of research regarding the organization, function, and defects of genes in this important region. The starting point for these projects has been the gene for coagulation factor VIII, which is responsible for the bleeding disorder hemophilia A.

I. Mutations in the Factor VIII Gene.

Hemophilia A is a relatively common inherited disorder, affecting 1 male in 5,000 worldwide. About one-third of hemophilia cases appear to be due to new mutations. Because of the clinical heterogeneity of hemophilia, a wide variety of mutations is expected. Thus investigating the origin and spectrum of hemophilia A–causing mutations should lead to a better understanding of mutagenesis in humans.

Because the factor VIII gene is very large (186 kb), a two-step approach has been taken to identify mutations in hemophilia DNA samples. A small region of interest is amplified from patient DNA by the polymerase chain reaction, and the amplified DNA is tested for mutations on a denaturing gradient gel. Variation in sequence changes the thermal stability of the molecule and therefore causes the amplified DNA fragment to shift in mobility on this type of gel, compared with that of a control sample amplified from normal DNA.

This approach was applied to the search for mutations in and near exon 8, which codes for one of the two acidic domains essential for proper factor VIII function. Screening of amplified DNA from 228 unselected hemophilia A patients revealed two new mutations, as well as a new diagnostic polymorphism in intron 7. Although technically more demanding, analysis by "heteroduplexing" amplified patient DNA samples with control DNA samples prior to electrophoresis should be more sensitive than analysis of simple "homoduplexes." Indeed, rescreening the same population by heteroduplexing revealed another mutation. All three mutations (two different missense and one 4 bp deletion) were observed in patients with clinically severe hemophilia and <1% factor VIII activity. Although only three mutations were found, only 3% of the mRNA and 0.16% of the gene have been examined.

Melt-map computer programs (provided by Dr. Leonard Lerman of the Massachusetts Institute of Technology) were used to calculate the theoretical shift in thermal stability for fragments with each of the mutations and the polymorphism. Generally there is good agreement between the predicted and observed mobility changes. These programs are now being used to help design a strategy for screening coding and flanking sequences throughout the factor VIII gene. In time this approach should uncover hemophilia-causing mutations in many patients. Knowledge of the underlying defect will greatly improve genetic counseling and aid in mutagenesis studies.

For example, the genetic origin of mutations was investigated in eight families with sporadic hemophilia whose defects had previously been determined by Southern blot analysis. Maternal mosaicism was discovered in two cases. In one case the mosaicism appeared to be restricted to the germline, but mosaicism clearly extended to somatic tissue in the second. This surprising result suggests that mosaicism may be a common feature of mutagenesis, and this possibility must be taken into account for genetic counseling.

II. Intron-22 Gene.

One remarkable feature of the factor VIII gene is the presence of a small gene completely contained within intron 22. This gene, which has no intervening sequences, is associated with a CpG island and a ubiquitous and abundant 1.8 kb transcript. Although the function of this gene is still unknown, several characteristics have come to light within the last year.

Two other copies of the intron-22 gene are present on the X chromosome. Preliminary mapping and sequencing suggest that they may be identical to the one in the factor VIII gene. Based on physical mapping studies (see below), they appear to be linked tightly to each other and lie within 1 Mbp of the factor VIII gene in the 5′ direction. Northern analysis of RNA from two hemophilia patients with independent deletions has shown that the intron-22 gene within factor VIII is transcriptionally active. Approximately 60% of intron-22 mRNA derives from the factor VIII gene itself, and the remaining 40%

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comes from one or both of the two extragenic copies.

Although the sequence contains a large open reading frame, initiated by a methionine, coding capacity may be a chance property of CG richness of the sequence. To address this issue, Dr. Gitschier (in collaboration with Dr. Glenn Hammonds at Genentech) has analyzed the sequence for coding likelihood. Two types of algorithms (Staden-McLachlan codon usage and Fickett test code) strongly predict that the sequence codes for a protein. The same algorithms applied to the scrambled sequence showed no protein-coding capacity. One unexpected outcome was the illustration of a possible frameshift in the coding sequence. Antibodies are being prepared to identify and isolate the protein and to resolve the frameshift question.

The intron-22 gene is present in a variety of mammals, although the gene in other species appears to be present in a single copy. Further studies in mice indicate that the murine counterpart is also transcribed and that it is not located within the factor VIII gene. Analysis of the murine intron-22 gene could be invaluable for ascribing a function.

III. Physical Map of Xq28.

Of the diseases that map to Xq28, the genes responsible for only three—hemophilia A, color blindness, and glucose-6-phosphate dehydrogenase deficiency—have been isolated. Accurate positioning of loci will be the first step in identifying the genes responsible for other Xq28 defects, such as adrenoleukodystrophy, Emery-Dreifuss muscular dystrophy, dyskeratosis congenita, nephrogenic diabetes insipidus, and spastic paraplegia, for which the biochemical basis is unknown. A number of studies have attempted to order the disease loci genetically using polymorphic DNA probes from Xq28, but the results are inconclusive.

Available DNA probes and cloned genes have been used to generate a large-scale physical map of Xq28 by pulsed-field gel electrophoresis. The resulting map covers 3 Mbp, estimated to be about one-half of the entire Xq28 region, and includes all probes and genes tested. Genes responsible for hemophilia A, glucose-6-phosphate dehydrogenase activity, and color blindness have been linked within 1.2 Mbp, and the direction of transcription for the former two has been established. A number of CpG islands have been identified; these may serve as tags for other genes in Xq28. This map will also provide the basis for systematically isolating new polymorphic DNA sequences needed for accurate genetic mapping in disease families.

One important question is the orientation of the map relative to the telomere and fragile site. Attempts are being made to incorporate the Xq28 telomere into an extended physical map. Also ongoing is a collaboration with Dr. Barbara Trask at Lawrence Livermore Laboratory to map the Xq28 sequences physically relative to the telomere by *in situ* hybridization with fluorescently labeled probes.

Dr. Gitschier is also Assistant Professor of Medicine (Genetics) at the University of California at San Francisco.

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REGULATION OF GENE EXPRESSION IN TRANSGENIC ANIMALS

ROBERT E. HAMMER, PH.D., *Assistant Investigator*

During the past year, research in Dr. Hammer's laboratory has continued to be focused on the regulation of eukaryotic gene expression. Specific areas of investigation include 1) the function(s) of lipoproteins and the low-density lipoprotein (LDL) receptor in the development of atherosclerosis, 2) the cis-acting DNA elements that direct and modulate cell-specific gene expression, and 3) oncogene-induced neoplasia of the cervix and pancreas.

I. Lipoproteins and Lipoprotein Receptors.

The LDL receptor is a cell surface receptor that plays an integral role in the metabolism of cholesterol in humans and animals. This receptor binds LDL, a plasma cholesterol transport protein, and carries it into cells. The LDL receptor is of primary importance in cholesterol metabolism, and consequently the development of atherosclerosis, and is a model system for the study of receptor-mediated endocytosis. During the past year, Dr. Hammer, in collaboration with Drs. Michael Brown and Joseph Goldstein (University of Texas Southwestern Medical Center at Dallas), has established lines of transgenic mice containing and expressing various human LDL receptor minigenes to investigate 1) the physiologic consequences of expressing the human LDL receptor gene when it is removed from its normal feedback control mechanisms, 2) the differences in LDL receptor internalization in various somatic tissues and, 3) the nature of receptor-mediated endocytosis of plasma lipoproteins by peritoneal macrophages, a cell type that is a major constituent of atherosclerotic plaque.

Mice containing fusion genes consisting of either the mouse metallothionein (MT) promoter or mouse transferrin promoter/enhancer fused to a human LDL receptor minigene were generated, and several lines of mice expressing high levels of human LDL receptor gene have been established. Mice containing either construct express the human receptor in a number of tissues, particularly the liver, and express the receptor to such levels that the animals chronically have little or no LDL in their bloodstream. Large numbers of mice exhibiting this phenotype are being generated and will be placed on high-cholesterol diets to examine how high internal stores of cholesterol affect endogenous LDL gene regulation and how such animals respond to these high-fat diets.

In addition to using these mice to investigate the effect of chronic overexpression of the LDL receptor, Dr. Hammer, in collaboration with Dr. Richard Anderson (University of Texas Southwestern Medical Center at Dallas), is using human LDL receptor mice to investigate how the LDL receptor functions in various animal tissues. Most tissues of the body require cholesterol for such housekeeping functions as membrane formation. Exactly how the LDL receptor contributes to cholesterol transport and cellular cholesterol homeostasis in each tissue is not known. The low expression of LDL receptors in the liver of untreated normal animals has made it difficult to study receptor function *in situ*. Immunocytochemistry is currently being utilized to map the distribution of the human receptor in the liver, kidney, and intestine of MT-LDL receptor transgenic mice.

Atherosclerotic plaques are filled with macrophages that have ingested large amounts of cholesterol and have been so stuffed with cholesterol esters that they have become converted into foam cells. Plasma lipoproteins are the primary source of the cholesterol found in these cells, but the molecular mechanism of lipoprotein uptake into macrophages is not clear. Recent studies have revealed that the LDL receptor is responsible for the uptake of β–very low density lipoprotein (β-VLDL) in mouse peritoneal macrophages. Because cellular uptake of β-VLDL is mediated through LDL receptors, it is important to understand the metabolism of the LDL receptor in macrophages. A fusion gene has been constructed that contains the human β_2-microglobulin gene enhancer/promoter directing the expression of the human LDL receptor minigene. Previous experiments in Dr. Hammer's laboratory have demonstrated that the β_2 promoter/enhancer directs expression to many cell types, including macrophages. Lines of mice containing and expressing this human β_2-LDL receptor gene have been established, and macrophages will be isolated from these transgenic mice and used to investigate lipoprotein uptake and LDL receptor metabolism in this important cell type.

During the past year a second area of investigation involving lipoproteins and the development of atherosclerosis has been initiated. Lp(a), a variant form of LDL, is present in the plasma of humans in amounts that vary from undetectable up to 100 mg/dl. High levels of Lp(a) are strongly correlated

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with atherosclerosis, although the mechanism by which it accelerates this process is obscure. In collaboration with the laboratory of Dr. Richard Lawn (Genentech), Dr. Hammer's laboratory is introducing fusion genes consisting of the mouse MT or transferrin promoter/enhancers and a human Lp(a) cDNA into the genome of mice. The intent is to express this foreign product in this species and use these mice to begin to investigate the role of Lp(a) in the progression of atherosclerosis.

II. Cell-specific Gene Expression.

A. *Rat elastase and trypsin genes.* The serine proteases are a family of digestive enzymes whose genes are expressed selectively in acinar cells of the exocrine pancreas. In collaboration with Dr. Ray MacDonald (University of Texas Southwestern Medical Center at Dallas), Dr. Hammer is continuing to characterize the regulatory domains within the elastase I gene and most recently the rat trypsin I gene that direct cell-specific expression.

The elastase I gene contains a 134 bp enhancer that, when fused to a human growth hormone (hGH) gene, is sufficient to direct high levels of pancreas-specific expression in an orientation-, distance-, and position-independent manner. This enhancer consists of at least three domains, any two of which are sufficient to direct acinar cell-specific expression in mice. Experiments are now in progress to determine the importance of each of these domains to cell-specific transcription.

To elucidate the cis- and trans-acting factors that regulate acinar cell-specific gene expression, Dr. Hammer and his colleagues have conducted experiments to define the pancreas-specific enhancer of trypsin I, another serine protease gene. Transgenic mice were made that contained one of four 5′-trimmed rat trypsin-hGH genes and were analyzed for growth hormone gene expression. Trypsin gene expression is controlled by a 229 bp regulatory element located between −225 and +4 of rat trypsin 5′-flanking DNA that confers cell-specific transcription. A smaller 130 bp fragment of rat trypsin I from −126 to +4 was unable to direct expression of the hGH gene. The 229 bp DNA fragment contains the putative pancreas-specific enhancer, which is shared among the pancreatic genes. Surprisingly, hGH expression was detected in the stomach of some transgenic mice bearing the rat trypsin-hGH gene. The pancreatic genes trypsin, amylase, and elastase were expressed in the glandular stomach, a cell type not previously known to produce these products. Experiments are in progress to define further the 5′ elements of other members of the serine protease gene family that direct acinar cell-specific gene transcription.

B. *Rat phosphoenolpyruvate carboxykinase (PEPCK) gene.* The cytosolic enzyme PEPCK is a pace-setting enzyme in gluconeogenesis, which is expressed primarily in the liver, kidney cortex, and adipose tissue. The synthesis of this enzyme is controlled in a differential manner in these three tissues. In liver, the levels of PEPCK are induced by cAMP and glucocorticoids and are decreased by insulin. In contrast, kidney PEPCK is induced by changes in acid-base balance and by glucocorticoids. Although many of the elements that are involved in hormonal and dietary modulation of PEPCK expression have been defined, little information has been available on the regulatory elements that direct the cell-specific and temporal modulation of PEPCK gene expression. In collaboration with Drs. Elmus Beale (Texas Tech University) and Mark Magnusson (Vanderbilt University), Dr. Hammer is identifying and characterizing some of the regulatory elements that direct the cell-specific transcription of the PEPCK gene.

Transgenic mice were made that contained one of five constructs consisting of various 5′ deletions of the rat PEPCK gene fused to an hGH gene. Transgenic animals containing these constructs are being screened for tissue-specific and developmental regulation of gene expression, as well as hormonal and dietary modulation of hGH expression.

III. Neoplasia.

A. *Cervical cancer.* Squamous cell carcinoma of the uterine cervix is one of the most common cancers among women. Correlation between human papilloma virus (HPV) infection of the uterine cervix and the development of cervical neoplasia is well established. HPV types 16 and 18 are found in more than 90% of all cervical tumors, suggesting that these viruses play a causative role in the development of this malignancy. Dr. Laimonis A. Laimins (HHMI, The University of Chicago) has demonstrated that the E6 and E7 gene products of HPV-16 and HPV-18 have transforming ability in NIH 3T3 cells and Rat-1 cells. Transgenic mice containing an HPV-18 construct have been made, in collaboration with Dr. Laimins, to determine the causal relationships between E6 and E7 expression and cervical carcinoma. Of 16 founder mice containing the HPV-

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18 transgene, 3 males showed enlarged seminal vesicles evident by 50 wk of age. One of these animals was bred, and a line has been established. Surprisingly, one female in the G1 generation developed a cervical tumor, evident at ~62 wk of age. Transgenic males and females in this line will be used to identify and characterize secondary events that lead to this transformation.

B. *Pancreatic cancer.* A mouse model for pancreatic acinar cell tumorigenesis has been derived by targeting the expression of the SV40 T antigen gene in transgenic mice by utilizing the rat trypsin I 5′-flanking DNA. The development of this tumor in mice bearing this construct is a multistep process that begins with pancreatic hyperplasia (evident before birth), which progresses to dysplasia and the formation of multiple large tumor nodules by 2–4 months of age. T antigen alone appears sufficient to induce hyperplasia, because all cells are affected similarly, and the effect occurs as early in development as T antigen can be detected. Because T antigen–induced hyperplasia rarely progresses to form tumors, other secondary events that complement or augment T antigen action must be required. Transgenic mice that reproducibly develop pancreatic adenocarcinoma should permit the identification of these secondary genetic events.

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REGULATION OF POSITIONAL INFORMATION IN *DROSOPHILA*

TULLE HAZELRIGG, PH.D., *Assistant Investigator*

The production of different cell types at particular places in the body depends on generalized signals, usually termed *positional information*. In *Drosophila*, positional information residing in the egg helps determine the initial distribution of cell types along both the anterior-posterior and dorsal-ventral axes. Later in development positional information is used to generate the normal patterns of gene expression within individual tissues. The long-term goal of research in Dr. Hazelrigg's laboratory is to understand how such positional information is established and how it instructs cells to assume their particular identities during development. The use of positional information is being studied in the early embryo and the developing eye.

I. Maternal Genes That Regulate Formation of Anterior-Posterior Polarity in the Developing Embryo.

Over the past few years, work in a number of laboratories has identified a set of maternal and zygotic genes that act in a hierarchical fashion to establish the normal anterior-posterior segmentation pattern of the embryo. The general model that has emerged is that maternal gene products, deposited in the developing oocyte, control the spatial pattern of expression of a set of zygotically acting genes (the gap genes) so that large, contiguous domains are established in the early embryo. Successively smaller developmental fields are established as the gap genes effect the expression of the pair-rule genes, which in turn may regulate the expression of the segment polarity genes. In concert with the action of the homeotic genes, this cascade of gene expression is necessary to establish anterior-posterior polarity, segmentation, and the assumption of segmental identities.

The key maternal component that initiates this cascade of anterior-posterior development is the product of the *bicoid* (*bcd*) gene. In newly deposited eggs, maternally expressed *bcd* RNA is present as a tight cap at the anterior end of the egg. During early cleavage divisions a steep gradient of *bcd* protein develops, with highest concentrations in the anterior-most 30% of the egg. The *bcd* protein gradient appears to be formed as a consequence of the translation of the localized *bcd* RNA, followed by diffusion. Thus the question of how developmental polarity is established becomes one of how the *bcd* RNA is localized within the egg. This question is part of the larger one of how developmental determinants are established within cells.

The maternal-effect gene *exuperantia* (*exu*) appears to be required for the proper positioning of the *bcd* RNA. In the absence of maternally encoded *exu* gene product, embryos die with loss of specific head structures. Gastrulation events, normally seen only at the posterior end of the embryo, occur also at the anterior end and are correlated with the formation of posterior structures at the anterior end. The *bcd* RNA is evenly distributed and not localized in eggs produced by mothers deficient for the *exu* gene. These observations demonstrate that the localization of the *bcd* RNA is a key event in determining the anterior-posterior polarity of the embryo and that the *exu* gene product is essential for localization.

In Dr. Hazelrigg's laboratory, the goal of work on the *exu* gene is to understand how the product of *exu* affects the anterior localization of the *bcd* RNA. Work has progressed on two fronts, genetic and molecular. X-ray mutagenesis screens for new *exu* alleles have yielded three mutations, two of which were small chromosomal deficiencies that localized *exu* to a region containing three polytene chromosome bands. Previous experiments had yielded a marked P-element insertion in one of these bands. Retrieval of the DNA flanking this P element provided the entry point for a chromosomal walk of 120 kb, which covered the region known to contain *exu* by the genetic analysis.

The location of the *exu* gene in this 120 kb walk has been determined by Southern blot analysis of DNA from mutant flies and from analysis of transcripts produced from this region. Particularly informative has been a hybrid-dysgenesis–induced mutation of the *exu* gene that contains a deletion of ~700 bp. Analysis of transcripts in this region in adults reveals a male-specific 2.6 kb mRNA and a female-specific 1.8 kb mRNA. Three *exu* mutants, including the one with the 700 bp deletion, are missing these transcripts.

Several *exu* cDNAs have been isolated from an ovary cDNA library and are being sequenced to determine the nature of the *exu* products. The one female cDNA that has been most extensively studied also hybridizes to the male transcript on Northern blots. Thus the differences in the sex-specific mRNAs appear to be due to alternative splicing of the *exu* transcript. cDNAs for the male-specific

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mRNA are being sought to confirm this hypothesis. Recently a number of genes in the sex-determination pathway in *Drosophila* have been implicated in the process of alternate splicing. The *exu* mRNAs may represent the first example of genes downstream of the sex-determining genes that are a target for alternative splicing.

The different sex-specific transcripts from *exu* are particularly interesting, given the finding that *exu* mutations affect spermatogenesis as well as oogenesis. Six of seven *exu* mutations cause male sterility. Abnormalities are seen in meiosis I of spermatocytes, and subsequent stages of sperm differentiation are disrupted. A detailed analysis of the precise defect in spermatogenesis is in progress.

The sequences of the cDNAs should help differentiate among a number of models of *exu* function. It is possible that different products are generated from the different mRNAs. Alternatively, the mRNAs may encode the same product in the two sexes. Even if the same product is made, it is not known whether it serves a similar function in the two sexes. One possibility is that the *exu* product localizes components within both spermatocytes and oocytes.

II. Genomic Position Effects That Alter the Expression of Adjacent Genes.

Expression of the *Drosophila white* gene is required for deposition of the red and brown pigments that produce the wild-type eye color of the adult. Usually the *white* gene is expressed in all regions of the eye, but in one case in which a P element carrying the *white* gene was inserted into the genome by transformation, the *white* gene shows a striking pattern of expression. In animals with this D-V insertion, *white* expression is limited to the ventral half of the eye. The D-V *white* gene contains all of the normal cis-acting regulatory sequences, since it is expressed throughout the eye when transposed to a new genomic location. Thus the D-V *white* expression appears to be repressed by flanking DNA in the dorsal half of the eye.

About 40 kb of DNA flanking the D-V element was cloned. Molecular analysis of five of six x-ray–induced revertant strains (where *white* is expressed throughout the eye) revealed rearrangements in either the 3′ or 5′ DNA flanking the D-V insertion. These data suggest that the repression exerted by the flanking DNA is not due to a single, simple enhancer sequence.

Under certain conditions the repressive action of the flanking DNA can extend into the ventral region of the eye. This extension occurs with a particular x-ray–induced derivative, D-V^{E42}, in which the eye has only a small quadrant of pigmented tissue. The D-V^{E42} mutation has recently been shown to delete ~3.5 kb of DNA overlapping the insertion site at the 5′ end of the D-V insertion. The same effect is seen when an allele of the *zeste* locus, z^1, is present in D-V flies. The product of z^1 can bind to the *white* promoter and has been hypothesized to act by looping DNA so as to bring together distant regulatory sites. Since the D-V^{E42} mutation removes DNA, the more severe phenotype may result from a similar juxtaposition of distant elements. This hypothesis predicts that there should be *zeste*-binding sites in the DNA flanking D-V and that a transcript from the region in wild-type flies should also be expressed differentially in the eye. Current efforts are directed toward confirming these predictions.

Dr. Hazelrigg is also Assistant Professor of Biology at the University of Utah.

PROKARYOTIC CHROMOSOME ORGANIZATION

DAVID R. HILLYARD, M.D., *Assistant Investigator*

I. Bacterial Genetics: Histone-like Proteins of Bacteria.

The organization of bacterial DNA into a highly condensed yet functional form is dependent on its interaction with a family of ubiquitous scaffolding proteins. Included among them is a class of small and usually basic proteins whose direct and indirect resemblance to eukaryotic histones has led to their being called histone-like proteins. Currently almost nothing is known about the role of these proteins *in vivo*. The major activity of this laboratory is to identify and manipulate the genes for this class of proteins in *Salmonella typhimurium*.

The most abundant of the histone-like proteins, HU, is extraordinarily conserved among bacteria. HU is able to bind and condense DNA, leading to the formation of nucleosome-like beaded structures. *In vitro* HU facilitates site-specific recombination needed for the inversion reaction of *Salmonella* flagellar phase variation, and it is essential for the transposition of bacteriophage Mu. The genes for the two *Salmonella* HU subunits, *hup*A and *hup*B, have been identified and inactivated. Surprisingly, the *hup*A, *hup*B double mutants are viable, indicating that HU is not an essential protein. However, gross alterations in the rates of *in vivo* Mu transposition, F plasmid stability, and flagellar phase variation are seen in cells that lack HU. When *hup*A and *hup*B mutant cells are tested, subunit-specific phenotypes are revealed. HU also seems to be required to maintain the normal distribution of supercoiled plasmid topoisomers. In addition, HU mutant strains overexpress a 17 kDa protein that has properties linking it to the family of histone-like proteins. The hypothesis that changes in the distribution of this protein or other type II DNA-binding proteins compensate for the loss of HU is being explored. An insertional mutation in a histone-like gene of *Salmonella* has recently been generated that results in an extreme growth-defective phenotype only in *hup*A, *hup*B double mutant backgrounds. In addition to HU, null mutations have now been generated for the *Salmonella hns* and *him*A genes, which also encode proteins of this class. The long-range goal is to use genetics to reveal interactive phenotypes among the extended family of bacterial histone-like proteins.

II. Conotoxin Genes.

Among venomous animals, cone snails have developed the capacity to envenomate an unusually broad diversity of natural prey successfully and in doing so have evolved an impressive array of toxins. Each species of *Conus* makes dozens of specific small toxic peptide ligands, which in many cases have high affinity to components of the nervous system. Little is known about the organization or evolution of toxin genes in any eukaryotic system. It is reasonable to think that the demands for toxin adaptation imposed on venomous animals may have led to novel genetic solutions. Work is continuing on the characterization of toxin genes from the mollusk-hunting snail, *Conus textile*. The full-length cDNA clones for the 27–amino acid King-Kong peptide have been obtained by screening cDNA libraries prepared from venom duct RNA. The sequence of the cDNA clone predicts an 80–amino acid propeptide with a single Lys-Arg processing site adjacent to the amino-terminal toxin amino acid. Using DNA probes specific for the amino-terminal portion of the propeptide, Dr. Hillyard's group has identified two additional *Conus textile* toxins. The structures of the three predicted propeptide molecules have been compared. In addition to a highly conserved amino-terminal region (which is presumably processed from the final toxin), the King-Kong peptides contain a perfectly conserved arrangement of six cysteines within the processed toxin segments. However, between cysteine residues there is absolutely no amino acid conservation. These cloning results and the fact that only a narrow range of cysteine arrangements occurs among conotoxin sequences suggest an unusual pattern of diversification for these molecules. In addition, these results suggest how conotoxin molecules specifically fold into one specific disulfide pattern (reduced and reoxidized peptide toxins assume many inactive configurations in addition to the active one). It is possible that information intrinsic to the conserved amino-terminal portion of the propeptide is needed to ensure specific folding.

Dr. Hillyard and his co-workers have recently constructed cDNA libraries from additional species of *Conus*, to expand the database to toxin families with dissimilar structural features and patterns of amino acid modification. In addition, work has begun on both the genetics and biochemistry of a

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novel *Conus* toxin that targets specifically to vertebrate NMDA (*N*-methyl-D-aspartate) receptors.

Dr. Hillyard is Assistant Professor of Pathology at the University of Utah School of Medicine.

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A COMPLEX FAMILY OF IMMUNOGLOBULIN HEAVY-CHAIN ENHANCER-BINDING PROTEINS

THOMAS R. KADESCH, PH.D., *Assistant Investigator*

During the past year, Dr. Kadesch's laboratory has used two genes to explore the nature of tissue-specific gene expression. The first gene, which commands the primary focus of the laboratory and is discussed in this report, is the immunoglobulin heavy-chain gene. It has been known for some time that B cell–specific expression of all immunoglobulin genes is controlled at the level of transcription. For the heavy-chain gene, much of this transcriptional control (activation) is thought to be due to a B cell–specific enhancer located in the intron between the J and C segments of the gene. The laboratory is attempting to identify and characterize the proteins that bind to the enhancer and mediate its activity. The second gene being studied is the human liver/bone/kidney alkaline phosphatase (LBK AP) gene. The LBK AP gene is also expressed at different levels in different cell types. It is expressed at very high levels in osteoblasts and at moderate levels in most other cell types. Hence the gene has two modes of expression: one (high) that reflects a specialized cell function (bone mineralization) and another (low) that possibly reflects a "housekeeping" function for the gene. The basic parameters that underlie this differential expression are being defined.

The efforts of many groups have led to the identification of several major protein-binding sites within the IgH enhancer. Most of these sites are important for enhancer activity. Hence the proteins that bind to them must be responsible for mediating enhancer function. Although this conclusion may be straightforward, the implications for the enhancer's cell-type specificity are not. Only one of the enhancer-binding proteins (Oct-2) is clearly restricted to B cells; the others appear to be present in a wide variety of cell types. Furthermore, the site that binds Oct-2 plays only a minor role in overall enhancer activity, and its absence does not affect cell-type-specific expression. The question remains: How is B cell–specific enhancer activity mediated by ubiquitous DNA-binding proteins? A recent series of experiments carried out in Dr. Kadesch's laboratory have begun to shed light on this issue. One approach has been to define systematically the protein-binding sites with regard to their influence on B cell–specific expression. Another has involved the direct cloning of cDNAs that encode enhancer-binding proteins.

Several years ago experiments with deletion mutants of the enhancer hinted that negative regulation may play at least a minor role in dictating B cell–specific expression. This has now been confirmed. Moreover, two protein-binding sites have been identified that recapitulate the initial results dramatically. One site, μE3, binds a ubiquitous DNA-binding protein. An oligonucleotide carrying only this site is capable of activating transcription from a linked promoter in both B cells and non–B cells. The other site, μE5, has no activity on its own. However, when the μE5 site is linked to the μE3 site (as it is normally found in the enhancer), activity is increased in B cells and totally abolished in non–B cells. Mutations introduced into the μE5 site restore expression in non–B cells. Hence the μE5 site stimulates activity of the μE3 site in B cells and inhibits its activity in non–B cells.

A cDNA clone, designated λ-3, that encodes a μE3-binding protein has been isolated from a B cell–derived cDNA library. The encoded protein binds to the μE3 site in a way that is indistinguishable from the binding activity seen with crude nuclear extracts. Furthermore, when this cDNA is overexpressed in mammalian cells, it specifically stimulates transcription of a reporter gene linked to a μE3-binding site. This confirms that the λ-3 cDNA encodes a μE3 transcription factor (TFE3). The predicted amino acid sequence of TFE3 identifies two regions that may specify protein oligomerization motifs. One is homologous to a region of c-*myc* and defines a putative helix-turn-helix motif. The other is a putative leucine zipper that lies adjacent to the helix-turn-helix region. Hence one question is whether TFE3 is able to form functional heterodimers with other related proteins. Heterodimer formation would not be required for activity of the TFE3 protein, however, because the transcription activation domain of the protein has been mapped to a region distinct from the presumed protein dimerization motif. Perhaps heterodimer formation is required for inhibiting the activity of TFE3.

Clones corresponding to μE5-binding proteins have also been isolated. The multiple cDNAs isolated thus far are encoded by at least three, and possibly four, distinct genes. Two of these have been isolated from a B cell–derived library and one (or two) from a non–B cell (HeLa) library. The two isolated from the B cell–derived library have been characterized in detail. Both are related at the level of amino acid sequence, suggesting that they are

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evolutionarily conserved. In addition, the two proteins are nearly identical in their *myc*-related helix-turn-helix motifs, which in turn are similar to that found in TFE3. This region is capable of directing the formation of heterodimers between the two different μE5-binding proteins. Each protein activates transcription as a GAL4 fusion protein, and the transcription activation domains determined in this manner also map to regions of the proteins that are distinct from their dimerization motifs. One of them is capable, when expressed on its own, of acting as a potent transcriptional activator in yeast.

The IgH enhancer has provided a fascinating model for tissue-specific transcription regulation. A ubiquitous positive transcription factor, TFE3, binds the enhancer next to a site that itself binds a family of proteins, one (or many) of which apparently represses the activity of TFE3 in non–B cells. It is likely that TFE3 makes contact with its neighbor proteins and that this interaction somehow helps mediate cell-type-specific activity of the enhancer. With cDNAs that encode these proteins now available, many of the aspects of this model can be tested directly.

Dr. Kadesch is also Assistant Professor of Human Genetics at the University of Pennsylvania School of Medicine.

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HUMAN GENETIC DISEASE

YUET WAI KAN, M.D., *Investigator*

The work in Dr. Kan's laboratory is focused primarily on the study of the pathophysiology and molecular diagnosis of genetic diseases of the red blood cell.

I. Abnormal Hemoglobins and Thalassemia.

A. *Prenatal diagnosis.* The method of prenatal diagnosis for sickle cell anemia and thalassemia continues to be refined, with the aim of simplifying it for routine use in areas where these diseases are common and automating the diagnostic procedure so that large numbers of screenings can be performed. Nonradioactive probes have been used to simplify the method and detect specific mutations in β-thalassemia. In a collaboration with researchers in China, horseradish peroxidase–labeled oligonucleotide probes were used to detect the common β-thalassemia mutations in southern China. Another nonradioactive method, denaturing gradient gel electrophoresis, identified all 12 β-thalassemia mutations described in the Chinese population. Because each mutation produces a distinctive band on the denaturing gradient gel, individuals heterozygous or homozygous for one mutation or doubly heterozygous for two mutations can be distinguished on the basis of their different patterns. This method is currently being tested in collaboration with investigators in Greece.

In a collaboration with Dr. Farid Chehab, a fluorescent label for the polymerase chain reaction (PCR) has been developed in which the primers used for PCR are labeled with one or more fluorescent dyes. Several different colored dyes have been used to detect gene deletions, chromosome rearrangements, and point mutations. The advantages of this method are that the result can be observed by eye immediately after amplification and the procedure lends itself to automation by real-time laser scanner on electrophoresis. The availability of an automated test would facilitate the implementation of the test in newborn screening programs. In addition to its application to genetic and neoplastic diseases, color PCR could also be applied to detect infectious agents.

B. *Spontaneous mutations in β-thalassemia.* The first case of spontaneous mutation in β-thalassemia was described 15 years ago. The β-globin gene from this patient has now been cloned and directly sequenced. Results indicate that the mutation occurs in the father's germline and is due to a single base deletion, resulting in a frameshift mutation at the codon.

C. *Globin gene expression.* The globin genes are expressed in a tissue-specific manner in erythroid cells. Four major hypersensitive sites (HS) upstream from the β-globin gene cluster enhance β-globin gene expression in erythroid cells, although their mode of action is not understood and is currently under investigation. One of the four sites, HSII, displays enhancer activity in transfection assays, as well as in transgenic mice. The activity was found to reside in a 700 bp fragment. Footprinting and gel mobility shift assays demonstrated protein binding to a direct repeat that contains the consensus sequence of an AP1 protein–binding site. Deletion of this site from the 700 bp fragment abolishes enhancer activity. Hence enhancement appears to be mediated through interaction with the AP1 protein–binding site. Further studies are in progress to characterize these protein interactions.

II. Other Red Cell Proteins.

A. *Protein 4.1.* In a collaboration with Drs. John Conboy and Mohandas Narla, the complex mechanism of alternate splicing in the protein 4.1 gene in erythroid cells continues to be studied. Protein 4.1 is a component of the cytoskeletal protein found in erythroid and nonerythroid cells. Many different isoforms of this protein are produced from a single gene by alternate splicing mechanisms that involve at least six different exons, two of which appear to be important functionally. One splicing involves a 21–amino acid coding block at the actin/spectrin-binding domain. The erythroid form of protein 4.1 includes these 21 amino acids, while the nonerythroid form does not. Another splicing occurs at the amino terminal, where the removal of 80 nucleotides upstream from the normal AUG and the addition of 17 nucleotides further upstream introduces a new AUG codon that adds 209 amino acids to the amino end of the protein. Antibodies prepared against the amino extension indicate that the protein is located in the cell nucleus. Further studies are in progress to determine its function.

B. *Glucose-6-phosphate dehydrogenase (G6PD).* The enzyme G6PD may be an example of a novel post-transcriptional modification of a gene product. It has been known for some time that G6PD is en-

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coded by a gene located on the long arm of the X chromosome. Studies on the red cell form of G6PD performed in Dr. Akira Yoshida's laboratory showed that the sequence of the amino end is not represented in the cDNA encoded by the X chromosome gene but in a different cDNA with a 345–amino acid open reading frame. Work from Dr. Kan's laboratory indicated that this cDNA is transcribed from a gene on chromosome 6. G6PD in the red cell is a combination of the chromosome 6 and chromosome X protein products. The chromosome 6 protein sequence switches to the chromosome X sequence at a methionine residue located 55 amino acids from the amino end of the chromosome 6 peptide. The mRNAs corresponding to the two species are 1.4 and 1.1 kb long, and no mRNA containing the combined sequence was found by Northern blot or by PCR, making the possibility of trans splicing unlikely. Either the two mRNAs are cross-translated so that the ribosomes jump from chromosome 6 mRNA to chromosome X mRNA at the position corresponding to the methionine residue or the two peptides are joined post-translationally by some as yet unknown mechanism. Further experiments are in progress to study these possibilities and to determine whether this phenomenon occurs in other cell systems.

Dr. Kan is also Professor of Laboratory Medicine and of Medicine and Louis K. Diamond Professor of Hematology at the University of California at San Francisco.

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MOLECULAR APPROACHES TO HUMAN NEUROMUSCULAR DISEASE

LOUIS M. KUNKEL, PH.D., *Associate Investigator*

The previous report detailed the complete cloning, sequencing, and identification of dystrophin, the protein product of the Duchenne/Becker muscular dystrophy (DMD/BMD) locus. This year has led to an increased understanding of dystrophin's function and how the absence of dystrophin in patients with dystrophin deficiency might be corrected. Dystrophin had previously been shown to be expressed in all types of muscle and the nervous system. Recent work has shown that the protein is slightly different in these tissues and may function differently in those tissues that produce dystrophin. The transcription start point is different for the nervous system than for muscle. This second start point lies at least 100 kb away from the muscle start point and has complete activity in *in vitro* assays. There is currently a search for patients who might have disruptions of this new first exon. Among those patients with X-linked mental retardation but no muscle weakness, there may be some who have mutations of this first exon.

One ongoing goal has been to relate molecular defects of the dystrophin gene with clinical symptoms observed in patients. Over 400 patients with DMD and the milder BMD have been studied at the molecular level; nearly 95% of patients fit the earlier theory of frameshift or nonframeshift deletion mutations. As part of this study an additional 15 exons from genomic DNA were sequenced, and oligonucleotides to detect more than 90% of all deletions by the rapid polymerase chain reaction have now been designed. Analysis of in-frame deletions found in BMD patients has established that the different domains of the dystrophin protein are more or less sensitive to disruption. Deletion mutations in the central portion of the gene are predicted to result in very mild to no symptoms at all—very different from classical DMD and BMD. Because of the association of cardiac problems with many of the DMD and BMD patients, a large collaborative effort has been established to screen males with cardiac problems for mutations in the central region, as well as other parts, of the dystrophin gene.

Concordant with experiments to understand more of dystrophin's function has been the isolation of proteins that are related in structure to dystrophin. These proteins may be capable of replacing dystrophin in DMD and BMD muscle and may interact with dystrophin in normal muscle. There is also another class of proteins that are part of the membrane cytoskeleton and might interact with or bind to dystrophin but are unrelated to dystrophin. Some of the relatives and binding proteins of dystrophin have been identified, and these are currently being isolated. It is hoped that this will lead to a better idea of the overall function of the membrane cytoskeleton in muscle and nerve. This line of experiments may have an additional benefit, in that these previously uncharacterized proteins are prime candidates to be disrupted in other neuromuscular diseases. Both DNA samples and tissues from patients who exhibit clinical symptoms that differ from DMD and BMD but might represent disruptions of the genes encoding the dystrophin-related or dystrophin-binding proteins are currently being banked by the laboratory.

The last area of research in which the laboratory has had some promising results concerns treatment of neuromuscular diseases. An animal model of dystrophin deficiency has been used, in collaboration with the laboratory of Dr. Terry Partridge in London, to transplant normal mouse muscle cells into the muscle of the dystrophin-deficient mouse. The input cells formed new muscle fibers and expressed dystrophin. The dystrophin was of normal size, location, and nearly 50% of normal levels. These promising experiments were done under special conditions; more experiments will be necessary to optimize the transplants so that they might be applied to preliminary human experiments. The transplantation of muscle cells may have far-reaching consequences for therapy for other disorders. Any metabolite produced by muscle that is affected by a genetic disorder might be corrected by cell transplantation.

In the next year, Dr. Kunkel's laboratory will attempt to address directly patient care and improved detection of the genetic disorders that affect both muscle and the nervous system.

Dr. Kunkel is also Associate Professor of Pediatrics at Harvard Medical School.

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MOLECULAR ANALYSIS OF BIRTH DEFECTS

DAVID M. KURNIT, M.D., PH.D., *Investigator*

I. Nondisjunction on Chromosome 21.

Dr. Kurnit's laboratory wishes to explore why the aging of mothers is associated with an increased risk for nondisjunction. In a five year effort supported by the National Institutes of Health, the centromere of chromosome 21 will be sandwiched between the cytogenetic markers on the short arm and DNA polymorphisms on the long arm. As their part in this program, Dr. Kurnit's laboratory is responsible for generating DNA sequence variation on 21q. In addition to the DNA polymorphisms for sequences on chromosome 21 that are available, the laboratory is also examining DNA sequence variation on chromosome 21 by using oligonucleotides that surround regions of local DNA sequence variation mediated by stretches of oligo d(A,C):d(G,T). Although it will take considerable effort to generate sequence variation on chromosome 21 using this strategy, once the oligonucleotides are constructed, DNA sequence variation in a large number of subjects can be examined rapidly. The two major advantages of this technique are that radioactivity need not be used and only a small amount of DNA is consumed. The latter point is important, because it obviates the need to transform white blood cells of subjects with Epstein-Barr virus to obtain sufficient quantities of DNA.

In collaboration with Terry Hassold in Atlanta, Dr. Kurnit and his colleagues have performed pilot studies to determine the parental origin of nondisjunction events that cause Down syndrome. This work demonstrates that crossing over occurs in a significant plurality (and perhaps all) cases of nondisjunction.

II. Genic Sequences on Chromosome 21.

Strategies have been employed to isolate expressed sequences on chromosome 21. Although the specific genes involved in Down syndrome have not been identified, their chromosomal location is known, as they have been mapped more precisely by other laboratories this past year to 21q22. The test for expression is presence of a homologous sequence in a complex cDNA library. A system (based on the πVX system of Brian Seed) has been developed and used to screen DNA probes for expression in an efficient manner. The πFlee series of vectors constructed in Dr. Kurnit's laboratory contain both the chloramphenicol gene and the *supF* gene. This allows harsher selection for integrates than was previously possible using *supF* alone. The amber plasmid vector Sumo 15A was designed by Dr. Kurnit for the purpose of constructing cDNA libraries and can be screened by recombination against πFlee clones containing pieces of DNA from regions of interest on chromosome 21.

Flow-sorted chromosome 21 fragments (courtesy of Drs. P. DeJong and H. Lehrach) have been shotgun cloned into cosmid vectors. After a cosmid is mapped to chromosome 21, it is fractionated and cloned into πFlee plasmids. Individual clones are grown up, and their copy number is corroborated, using the recombination-based assay. Single-copy clones carrying fragments of genomic chromosome 21 DNA are infected with Sumo 15A–cDNA phage. The lysate is plated on DK21, an *Escherichia coli dnaB* amber host that was constructed by Dr. Kurnit's laboratory and that selects for phage carrying *supF*. Only phage containing *supF* can grow in the cell line DK21, and the only way *supF* can have been acquired is by homologous recombination. Thus a method has been established for the isolation of expressed sequences on chromosome 21.

III. Situs Inversus in Mouse.

The *iv* mutation in mouse is an autosomal recessive mutation that causes random determination of situs for the heart and other internal organs. Dr. Kurnit's laboratory (with Dr. W. Layton) is crossing inbred strains containing this mutation with other mouse strains to establish linkage with the mutation. A recent report (*Proc Natl Acad Sci USA* 86:5035–5038) mapped this mutation to mouse chromosome 12. With a larger series of animals, Dr. Kurnit and his colleagues confirmed this report and showed that the *iv* mutation is very close (<1 centimorgan) to the immunoglobulin heavy-chain locus on this chromosome. Genomic strategies to walk to the *iv* locus from the mouse Ig-H locus can now be considered.

IV. Two-dimensional Protein Gels and Mouse Database.

Major organogenesis takes place during days 7 to 10 of gestation in the mouse. Dr. Kurnit has created a database of embryonic proteins synthesized dur-

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ing this critical window of development by producing two-dimensional gel electrophoresis protein profiles of embryos at half-day intervals (days 7 to 10). This resource provides a basis of comparison for examination of effects of genetic mutation or mutagenic/teratogenic agents. Dr. Kurnit has shown that there is a unique protein pattern for each half-day time point, with a burst of novel protein synthesis on day 8. This database is being used to examine proteins affected by the *iv* murine mutation, which are therefore potential candidates for playing a role in determination of laterality and spatial interrelationships of the internal organs. The effects of the mutagen retinoic acid, which produces situs inversus similar to the *iv* mutation as one of its effects, are also being examined with this database. Multiple quantitative alterations caused by the *iv* gene on day 8.5 have been documented, and several of these overlap alterations caused by a retinoic acid phenocopy of the *iv* gene.

Dr. Kurnit is also Professor of Pediatrics and Human Genetics at the University of Michigan Medical School.

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HUMAN PAPILLOMAVIRUSES

LAIMONIS A. LAIMINS, PH.D., *Assistant Investigator*

Dr. Laimins is studying the molecular biology of human papillomaviruses (HPVs). Areas of interest include 1) transformation of human epithelial cells, 2) structure and function of viral gene products, 3) regulation of viral gene expression, and 4) transgenic mouse models for HPV-induced malignancy.

Papillomaviruses are small DNA viruses that are responsible for a wide range of benign tumors in humans and other animal species. Subtypes of the HPVs are the presumed etiological agents of carcinomas of the cervix and penis. HPV types 16 and 18 are found in more than 90% of all cervical tumors, suggesting that papillomaviruses may play a causative role in the development of these malignancies. In the majority of malignancies, some or all copies of the HPV genomes are found integrated into the host chromosome, while in benign lesions the virus remains episomal. In the integrated state only a subset of all viral gene products is expressed. One model for HPV-induced malignancy proposes that integration is an activation event for transformation.

I. Transformation and Transforming Proteins.

Dr. Laimins and his colleagues are using primary human keratinocytes to study HPV transformation *in vitro*. Previous studies in rodent fibroblasts have shown that both the E6 and E7 gene products have independent transformation activities, but only one gene is necessary for the transformation of immortalized rodent fibroblasts. Studies in human keratinocytes, the natural host cells for HPV infection *in vivo*, have demonstrated that both E6 and E7 are required for high-frequency transformation of keratinocytes. The ability to immortalize and alter differentiation *in vitro* has been used as a criterion for transformation.

An *in vitro* system for the differentiation of epithelial cells involving growth of cells on collagen at an air-to-liquid interface has been used by Dr. Laimins and his colleagues to assay for altered differentiation *in vitro*. This system has been used to duplicate the histological changes seen in cervical cancers *in vivo*. A common characteristic of cervical tumors is a lack of epithelial differentiation, and cancers can be thought of as composed of cells that do not undergo terminal differentiation. The E7 gene product alone can, at a low frequency, immortalize keratinocytes but has little effect on differentiation. High-frequency transformation, as indicated by an altered pattern of differentiation, appears to require the synergistic action of both the E6 and E7 gene products.

Recombinant baculoviruses have been used to synthesize large quantities of the E6 and E7 open reading frames in insect cells in order to understand the molecular mechanisms by which HPV E6 and E7 gene products act to transform cells. HPV-18 E6 protein synthesized by recombinant baculoviruses has been localized to the nucleus of infected cells and appears to be a DNA-binding protein. In addition, Dr. Laimins and his colleagues have determined that E6 binds zinc and is thus a member of the zinc finger family of proteins. However, E6 is an unusual zinc finger protein, as it has 30 amino acid residues separating finger motifs, as opposed to the usual 15 residues. It also has been shown that the stability of the E6 protein is determined, at least in part, by its amino-terminal amino acids.

II. Regulation of Viral Gene Expression.

Dr. Laimins and his colleagues are characterizing the cis and trans elements responsible for regulation of HPV expression. Three viral enhancer elements have been identified, and two were found to be responsive to papillomavirus trans-acting factors. The third element functions as a constitutive enhancer and requires only cellular factors for function. The first enhancer (IE2) is responsive to a papillomavirus E2 protein, while the second enhancer (IE6) is responsive to the E6 gene product. The constitutive enhancer functions in a wide variety of cell types but exhibits a cell-type preference for epithelial cells. The cellular factors involved in the action of this enhancer are being characterized. The IE2 enhancer is probably the principal enhancer active in benign lesions, where the virus exists as an episome. In the integrated state, E2 expression is disrupted and the constitutive enhancer becomes most active. This change in transcriptional patterns may play an important role in the progression of the lesion to a malignant state. The E2 protein has been overproduced in baculovirus-infected cells, and the biochemistry of this protein is being studied in *in vitro* systems. In additional studies, the laboratory has shown that viral transactivators from herpes simplex virus (HSV) may also activate

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HPV expression, implicating HSV as a possible cofactor in HPV-induced disease. In addition, the phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate (TPA) also has been shown to increase HPV-18 expression, suggesting chemical cofactors also may act to alter papillomavirus transcription.

III. Transgenic Models for HPV-16– and HPV-18–Induced Disease.

A series of HPV-16 and HPV-18 transgenic mice have been constructed, in collaboration with Dr. Robert E. Hammer (HHMI, University of Texas Southwestern Medical Center at Dallas), in an attempt to show a causal relationship between HPV and epithelial tumors. After 10 months, 25% of male founder mice expressing the E6-E7 region alone, developed hyperplastic seminal vesicles. This phenotype has been transmitted through the germline and appears to be linked to expression of the E6-E7 region of HPV-18.

Dr. Laimins is also Assistant Professor of Molecular Genetics and Cell Biology and on the Committee on Virology at The University of Chicago.

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GENETIC INVESTIGATION OF COMPLEX INHERITANCE

JEAN-MARC LALOUEL, M.D., D.SC., *Investigator*

Research in Dr. Lalouel's laboratory focuses on two topics: 1) the identification of genetic factors that account for familial aggregation of disorders of lipid metabolism and early occurrences of myocardial infarction and 2) the construction of genetic maps of human chromosomes, a project pursued in collaboration with Dr. Raymond L. White (HHMI, University of Utah). The major focus of research during the past year has been an investigation of lipoprotein lipase (LPL) deficiency in relation to hypertriglyceridemia.

Complex lipoprotein phenotypes are commonly observed among relatives of patients with early coronary disease, with various patterns of hypercholesterolemia and/or hypertriglyceridemia. Complex lipoprotein profiles have been considered to result either from the variable expression of a single-gene defect or from the independent contribution of two or more genes, but etiological heterogeneity further confuses the issue. The investigation of a large kindred expressing complex lipoprotein phenotypes, K659, yielded preliminary results in support of the second hypothesis: one gene accounted for hypercholesterolemia, while hypertriglyceridemia depended on other factors. Because chylomicronemia was noted in one pedigree member, LPL, which is a key enzyme in the delivery of fatty acids to peripheral tissues, stood as a possible candidate for the cause of the defect. Deficient LPL activity is found in a rare form of familial hyperchylomicronemia that is inherited as an autosomal recessive trait. However, the heterozygous state remains poorly characterized. Are heterozygotes particularly prone to hypertriglyceridemia? Can heterozygosity for a defect at the LPL locus account for some of the commonly observed forms of familial hypertriglyceridemia?

Another pedigree, K2003, ascertained through a patient with documented deficiency of LPL activity, was investigated in an effort to address the above questions. In collaboration with Dr. Lalouel's group, Dr. P. H. Iverius at the Veterans Administration Hospital in Salt Lake City confirmed by immunoassay that LPL was present in this proband. The sequence of human LPL cDNA had just been reported in the literature, but no mutation had been identified. The mRNA prepared from adipose tissue of this patient was used to synthesize cDNA, from which the complete coding region of LPL was amplified and cloned. Eight independent clones were sequenced. A single-nucleotide difference was identified in the patient's LPL cDNA, leading to the substitution of a glutamic acid for a glycine. The patient was found to be homozygous for this mutation by dot-blot hybridization with specific oligonucleotides, and both parents were confirmed heterozygotes. To establish the functional significance of this molecular variant, the mutation was reproduced by oligonucleotide-directed *in vitro* mutagenesis, and transient expression of both normal and mutant genes was achieved by transfection of COS cells after cloning into the expression vector pSVL. Normal activity was recovered both in medium and in cell extracts after transfection with the normal sequence; no LPL activity could be demonstrated after transfection with the mutant sequence, although concentrations of immunoreactive material were similar to those obtained with the normal sequence. These results confirmed that the mutation identified in the patient led to the production of an inactive product.

The identification of this mutation permitted determination of the genotype of relatives of the proband by hybridization of their individual genomic DNAs, amplified by the polymerase chain reaction, to specific oligonucleotides; it also permitted testing for a relationship between carrier status for the mutation and the presence of hypertriglyceridemia noted in multiple members of the pedigree. An association was found in an initial screen of 27 relatives of the proband; this association was confirmed by extending the original pedigree to include 61 relatives. However, only 50–60% of the carriers of this mutation expressed hypertriglyceridemia. Therefore, although individuals heterozygous for an allele encoding a functionally deficient LPL enzyme were prone to exhibit hypertriglyceridemia, it appeared that other factors were necessary to induce the expression of this phenotype. These findings may be relevant to hypotheses being advanced for the inheritance of familial hypertriglyceridemia and related disorders.

The hypothesis was subsequently entertained that the chylomicronemia observed in one member of pedigree K659 may result from LPL deficiency. Dr. Iverius established that LPL activity in the plasma of this subject after heparin injection was below the fifth percentile of normal controls. The strategy outlined above for K2003, applied to clone and sequence the entire coding region of the LPL gene in this subject, led to the identification of an-

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other single–amino acid substitution in one allele. This mutation was confirmed by analysis of genomic DNA, produced *in vitro* and expressed transiently in COS cells. No LPL activity was detected either in medium or in cell extracts, and results of immunoassay are pending; therefore no conclusion can yet be made about the significance of the mutation in this patient.

These findings have spawned a particular interest in the molecular genetics of LPL. Analysis of the LPL gene has been initiated in a series of patients to identify new mutations, test their functional significance after transient expression, and use these naturally occurring events to investigate the structure and function of this enzyme.

Dr. Lalouel is also Professor of Human Genetics at the University of Utah School of Medicine.

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MOLECULAR CYTOGENETICS

LABORATORY OF THE LATE SAMUEL A. LATT, M.D., PH.D., *Investigator*
BY MARC LALANDE, PH.D., *Senior Associate*

I. Molecular Analysis of the Human 15q11q13 Subregion.

Dr. Lalande is continuing the pioneering work of Dr. Latt on molecular studies of two distinct human genetic disorders, Prader-Willi syndrome (PWS) and Angelman syndrome (AS), both of which are associated with abnormalities of the 15q11q13 chromosomal subregion. Ten cloned DNA markers specific for this region have been isolated from a flow-sorted recombinant DNA library. Five of these have been mapped to a subregion of 15q11q13 that is deleted in 60% of patients suffering from PWS. In collaboration with Dr. Joan Knoll, it has been demonstrated that the similar region of 15q11q13 is also deleted in a number of AS patients. Although the deletions are indistinguishable at the molecular and cytological levels, the clinical phenotypes of the two disorders are different. The clinical features of PWS include obesity, small hands and feet, hypogonadism, hypotonia, growth delay in infancy, and mild mental retardation; AS is characterized by severe mental retardation, microcephaly, seizures, prognathism with tongue protrusion, and puppet-like ataxic gait with jerky arm movements. Using restriction fragment length polymorphisms characterized by Dr. Robert D. Nicholls, Dr. Knoll has determined that in each of the seven AS cases studied, the deleted chromosome is inherited from the mother. This is in contrast to PWS, where the deleted chromosome is paternally inherited. These results suggest that the sex of the parent who transmits the abnormal chromosome plays a role in determining the clinical phenotype, i.e., AS or PWS.

These studies have now been extended to the analysis of PWS patients who display an apparently normal karyotype, in that no deletion of 15q11q13 can be detected at either the molecular or cytogenetic level. Drs. Nicholls, Knoll, and Lalande have shown in the first two families studied that the PWS patient inherits both of the mother's normal and intact (at least for the 15q11q13 critical region) chromosome 15s. This is the first time that the inheritance of both the normal chromosomes from one parent in an individual has been demonstrated in humans and the first time that such an anomaly has been associated with a genetic disease (PWS). It appears that a gene or genes within 15q11q13 are required for normal human development. The absence of a paternal contribution of genes to this region, whether by deletion of the paternal 15q11q13 or by inheritance of two maternal chromosome 15s will result in PWS. This implies that there are functional differences in alleles of a gene or genes from 15q11q13 that depend upon the sex of the transmitting parent. This phenomenon is termed *genetic imprinting*. Future studies will concentrate on the possible imprinting mechanism and the role of imprinting in PWS, AS, and, perhaps, other human genetic disorders.

II. Chromosome Mapping Studies.

Dr. Latt's laboratory continues to generate DNA fragments specific for several important chromosomal regions and to analyze these subregions using various molecular techniques. In collaboration with Dr. Matthew L. Warman, a physical map of ~1 million base pairs (bp) of DNA surrounding the oncogene N-*myc* was constructed in normal cell lines. This map was compared with that of neuroblastoma tumor cell lines, where the N-*myc* oncogene is specifically amplified. Complex rearrangements in the amplified N-*myc* locus of tumor cells were observed that may be important in understanding the process of neoplastic change and tumor progression. In collaboration with Dr. Ulrich Müller, a physical map of almost the entire short arm of the human Y chromosome has been constructed. The map encompasses ~13 million bp and was generated using a number of Y chromosome–specific DNA fragments isolated from flow-sorted recombinant DNA libraries and a one-dimensional pulsed-field gel electrophoresis system. Similar techniques have been employed to extend the physical map of human chromosome 13. This map now spans ~12 million bp of the 13q14 subregion, which contains the retinoblastoma susceptibility (RB) gene locus. Rearrangements, such as deletions and translocations, that involve the RB gene can be detected using conventional as well as long-range mapping techniques and DNA fragments cloned in Dr. Latt's laboratory. The RB gene that was originally isolated using such fragments has now been shown to be involved in the development of several different human tumors, including breast and colon cancer.

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III. DNA Replication Studies.

Dr. Lalande is working with a new class of compounds that inhibit cell growth. These compounds, which include the plant amino acid mimosine, arrest the cell cycle in the late G1 phase prior to the onset of DNA synthesis. No cell cycle inhibitors that act at this point in the cell cycle had previously been described. The mimosine and related compounds will, therefore, offer a unique tool for analyzing the key biochemical events that control the initiation of DNA synthesis in mammalian cells.

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MOLECULAR GENETICS OF MAMMALIAN TESTIS DETERMINATION AND SPERMATOGENESIS

YUN-FAI CHRIS LAU, PH.D., *Associate Investigator*

Dr. Lau's laboratory is studying the molecular genetics of mammalian sex determination, testicular differentiation, and spermatogenic regulation. For the past year, Dr. Lau and his associates have focused on evaluating these biological processes through two candidate genes, the zinc finger Y (*ZFY*) and the male-enhanced antigen (*MEA*) genes.

I. Characterization of the *ZFY* and Related Genes.

The *ZFY* gene is a candidate for the testis-determining factor gene (*TDF*) on the human Y chromosome and is postulated to initiate testis differentiation during embryogenesis. However, *ZFX*, a gene that is highly homologous to the *ZFY* gene, has also been identified and mapped to the p21 band of the human X chromosome. Southern analysis indicated that both *ZFY* and *ZFX* genes are present on the sex chromosomes of most eutherian mammals. The identification of the *ZFX* gene on the X chromosome has made it difficult to establish the dominant role of the *ZFY* gene in sex determination.

A. ZFY *encodes a protein with two domains in the adult human testis.* A full-length *ZFY* cDNA was isolated from an adult human testis cDNA library. Sequence analysis indicated that the corresponding transcript encodes a 90.6 kDa protein of 801 amino acids. The ZFY protein is composed of two domains. The carboxyl-terminal end consists of 13 zinc finger repeats, similar in structure to other DNA-binding proteins involved in regulating the transcription of other genes. The amino-terminal end of the protein is highly acidic and negatively charged. A short stretch of basic amino acids seems to define the boundary of these two domains, and other investigators have postulated this to be the nuclear localization signal. Sequence analysis of a partial cDNA from the *ZFX* gene revealed that *ZFY* and *ZFX* genes share 1) 95% and 97.4% homology at the DNA and protein levels, respectively, at their zinc finger domains and 2) a 94% homology at both DNA and protein levels at their acidic domains.

B. *Human* ZFY *and* ZFX *genes are differentially expressed in adult gonadal and somatic tissues.* Expression analysis indicated that the *ZFY* gene is transcribed primarily as a 3 kb mRNA in adult testis and frequently as a 5.7 kb transcript in male tumor cell lines of various somatic origins. The *ZFX* gene produces two major transcripts of 6.7 and 8.0 kb in ovaries and somatic tissues and cell lines of both sexes. In the somatic tissues analyzed, the *ZFX* transcripts are the predominant mRNA species; the *ZFY* transcript is scarcely detectable. The differential expression of both genes indicates that they may not be functionally identical and may serve separate biological functions. Significantly, the 3 kb *ZFY* transcript is also detected in other mammalian adult testes, suggesting that it may play an important role in the normal physiology of this organ.

C. *Expression of the* Zfy *genes in adult and fetal mice.* The mouse harbors two *Zfy* genes, *Zfy-1* and *Zfy-2*, in the sex-determining region of its Y chromosome. Only *Zfy-1* is needed for testis development, and it is considered a candidate gene for the testis-determining Y (*Tdy*) locus. In the adult, expression of both *Zfy* genes can only be detected in the testis as a major 3 kb and a minor 2.7 kb transcript. The transcription increases with the initiation of meiosis and achieves the highest level in the round spermatids. Differential expression of these two genes was observed: the expression of the *Zfy-2* gene was slightly greater than that of the *Zfy-1* gene. In fetuses, low levels of *Zfy* expression were detected in several tissues, including testes, at day 12 of gestation. In contrast, in adults the expression of the *Zfy-1* gene was greater than that of the *Zfy-2* gene. The data demonstrating the *Zfy-1* gene expression in fetal testes support the hypothesis that this gene plays a role in testis differentiation. However, since the *Zfy* genes are also expressed during spermatogenesis and in fetal organs other than testes, they may play other roles, such as regulation of male germ cell development, in addition to their postulated role in testis determination.

D. Zfy *genes are silent in adult XY ovary.* Are high levels of *Zfy* transcription restricted to testes, or can these Y-encoded genes also be expressed in XY ovaries? To answer this question, Dr. Lau and his colleagues performed expression studies of these genes on the gonads of the B6.Y^{Dom} strain, which exhibits XY sex reversal. XY sex reversal occurs when the Y chromosome of certain *Mus musculus domesticus* strains is introduced into the B6 strain. Half of the XY progeny develop bilateral ovaries,

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while the remainder develop varying degrees of ovotestes. This XY sex reversal has been postulated to be the result of incompatible interactions between the *domesticus Tdy* and other autosomal and/or X-linked testis-determining genes. Results from these studies indicated that the *Zfy* genes are only expressed in the testes but not in ovotestes or ovaries from the same individuals, thus confirming the linkage of *Zfy* gene expression to spermatogenesis. In addition, the data demonstrate that the *Zfy* genes are silent in adult XY ovarian environment.

E. *Possible involvement of the* Zfx *gene in sex determination.* Sex reversal occurs naturally in the Scandinavian rodent, the wood lemming, due to mutations in a variant X* chromosome, such that X*Y individuals develop into fertile females. Chromosome mapping of homologous sequences (*Zfy* and *Zfx*) to the human *ZFY* gene has localized the *Zfx* sequence on bands p11–12 of both the normal X and variant X* chromosomes, at or proximal to a presumed breakpoint (p12) involved in the generation of X* from X. Molecular differences between the *Zfx* and *Zfx** sequences are readily detected by Southern hybridization, suggesting that the *Zfx* gene might have been altered by such genetic rearrangements. Furthermore, at least 15 copies of the *Zfy* sequences are distributed along the entire short arm of the Y chromosome. Multiple *Zfy* sequences have also been demonstrated on the Y chromosome of a South American rodent, *Akodon azarae*, in which XY sex reversal also occurs naturally in both the wild and laboratory stocks. The multiplicity of the *Zfy* sequences hence seems to be a common factor in these two species with XY females. If the *Zfy* gene(s) is indeed the testis-determining gene, the present observations suggest 1) the *Zfx* gene probably interacts with the *Zfy* gene(s) in testis determination, 2) the mutated *Zfx** gene becomes incompatible in such an interaction, and 3) the multiple *Zfy* sequences may potentiate the sex reversal in the X*Y animals.

II. Characterization of the Male-Enhanced Antigen (*MEA*) and Related Genes.

The *MEA* gene was initially isolated with a pool of specific antisera against the serological H-Y antigen. Molecular characterization established that the *MEA* gene is phylogenetically conserved and expressed at high levels in adult testes, particularly in round spermatids. Two linked genes, *Gene A* and *B*, have been identified within 60 kb of human genomic DNA. The structural genes for both *MEA* and *Gene A* are present within a 40 kb human insert of a recombinant cosmid, CosMEA-A. The promoters of both *MEA* and *Gene A* are arranged in a head-to-head manner, such that their putative start sites are only separated by 450 bp of GC-rich sequence. This common promoter contains no consensus TATA or CCAAT boxes and is capable of mediating the expression of a reporter gene, chloramphenicol acetyltransferase (CAT), in a bidirectional manner. Preliminary analysis has identified a small segment of ~100 bp, defined by two putative DNase I–hypersensitive sites, within this promoter sequence that interacts specifically with testicular protein extracts in a gel-retardation assay. These results suggest the possible existence of tissue-specific factor(s) regulating the expression of both *MEA* and *Gene A* in the testis.

Five transgenic mice harboring the entire CosMEA-A cosmid were generated. The human *MEA* and *Gene A* in the offspring of these founders were expressed in a tissue-specific manner, similar to those of the endogenous mouse genes. These results suggest that the 450 bp promoter segment may be important in mediating the tissue-specific expression of the human *MEA* gene and *Gene A* in these transgenic mice and support the hypothesis that the *MEA* and linked genes serve an important role(s) in mammalian spermatogenesis.

Dr. Lau is also Assistant Professor in the Departments of Physiology and Medicine at the University of California at San Francisco.

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GENETIC CONTROL OF CELL GROWTH AND DEVELOPMENT

PHILIP LEDER, M.D., *Senior Investigator*

Cell growth and development are precisely ordered biologic processes under the control of a complex genetic program. Dr. Leder and his colleagues have undertaken to understand aspects of this program at the molecular genetic level.

I. Genetic Control and Modulation of Oncogenesis.

A. *Nonclassical genetic control of oncogenesis.* Certain human malignancies tend to be highly prevalent in families, leading to the notion that they are controlled by an array of genes, rather than by a single Mendelian locus. Such inheritance patterns are called polygenic. Although certain of these genetic elements could be classical oncogenes, the nature of oncogenesis suggests that tumorigenesis can also be influenced by complex features of an organism, such as cell population dynamics, host defense mechanisms, physical barriers to tumor growth and vascularization, DNA repair mechanisms, and other genetically controlled processes that are specified by polymorphic alleles within human populations.

Since many malignancies are characterized by the specific cell type in which they occur (e.g., Burkitt lymphoma occurs in the B cell stage of immunocyte development), the genetically programmed developmental pathway followed by a cell might also influence the occurrence of cancer within that cellular lineage. That is, the developmental program produces the specific cells "at risk" for malignant transformation. Dr. Leder and his colleagues have tested this by producing a strain of transgenic mice that develop a predictable and reproducible form of pre–B cell lymphoma. The pathway of immunocyte development in this test line was altered by breeding into it a transgene from a second strain of transgenic mice. The second strain carried a transgene directing the synthesis of an assembled, membrane-bound immunoglobulin heavy chain. The net effect of introducing the complete immunoglobulin heavy-chain transgene is to allow a developing immunocyte to bypass the immunoglobulin gene assembly stage in B cell development. Bypassing this stage of B cell development should reduce or eliminate the population of developing B cells at the cancer-prone stage of their development and thus reduce the population of cells at risk. As a consequence, the immunoglobulin transgene should reduce the incidence of cancer in the test line.

The immunoglobulin transgene dramatically reduced the incidence of pre–B lymphoma in the test strain. In essence, the immunoglobulin transgene behaved as an anti-oncogene, but, instead of modulating transformation at the cellular level, it modulated cancer incidence at the level of the organism by perturbing a developmental pathway. It reduced the risk of cancer in the cancer-prone strain.

B. *Antitumor effects of biologic response modification.* Dr. Leder's study of complex factors that can modulate tumor incidence suggested that specific biologic effectors influencing cell differentiation could also exert profound effects on carcinogenesis. The cytokines are a group of protein factors elaborated by cells that appear to effect cellular growth and development. Interleukin-4 (IL-4), one of the cytokines described to date, has the ability to induce the maturation and class switching of B cells). An effort was made to create transgenic mice in which the expression of IL-4 was placed under the control of immunoglobulin promoter/enhancer sequences, so that its effects on the development of lymphomas could be assessed. Transgenic mice carrying the IL-4 construct exhibited an unusual phenotype (currently under investigation) that, briefly stated, aborts the development of thymus-dependent T cells and ultimately proves lethal.

Because of the effect of the transgene on the developing immune system, another more effective means of assessing the antitumor effects of cytokines was designed to detect antitumor activity that depends on the mobilization of host defenses. Malignant cells were transfected to produce (in this case) IL-4, and the ability of the transfected, IL-4–producing tumor to grow in syngeneic mice was measured. The effect of the cytokine could be further assayed by mixing the IL-4–producing cells with nonproducing tumor cells of a variety of origins. Dr. Leder and his colleagues were able to show that IL-4 has a potent, non-cell-autonomous, *in vivo* antitumor effect. This activity, which is thymus-independent, appears to be mediated by an inflammatory infiltrate. This result targets IL-4 for further attention as an agent for potential use in the treatment of human malignancy.

II. Transgenic Approach to Embryonic Development.

Studies in Dr. Leder's laboratory and elsewhere have shown the great potential of the transgenic mouse system to perturb developmental processes. Yet another, and even more instructive, means by

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which this perturbation comes about is through the insertional mutation of genes required for normal morphologic development. Dr. Leder and his colleagues previously produced a transgenic insertional mutant that is defective in the normal pathway of limb development. Investigations of this mutant, *limb deformity* (*ld*), have led to the recent discovery a new *ld* allele, a suppressor mutation, *lst*, and to the cloning of the *ld* gene. A systematic effort has been made to discover new insertional mutants among the transgenic mice made for other experimental purposes.

As the result of a systematic program of intercrossing transgenic animals, a new mutation for perinatal lethality was discovered and, using classical genetic techniques as well as *in situ* analysis, was mapped to the distal portion of chromosome 15. Homozygous animals are born and initially appear normal, but die within 24 h; hence the name *transgenic perinatal lethality* (*Tg.ple*). Since the inserted fragment of DNA marks the locus at the molecular level, it should be possible to identify the responsible gene and understand the basis of this lethality, and thus gain insight into a potential cause of fetal wastage.

Dr. Leder is also John Emory Andrus Professor of Genetics at the Harvard Medical School.

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MOLECULAR GENETIC APPROACHES TO METABOLISM AND METABOLIC DISEASES

FRED D. LEDLEY, M.D., *Assistant Investigator*

This laboratory is involved in molecular genetic studies of methylmalonyl CoA mutase (MCM) and its deficiency state in humans, methylmalonic acidemia (MMA). This enzyme provides a system in which to investigate several areas of biological, evolutionary, and medical significance. Previous work in this laboratory identified a full-length human MCM cDNA clone that provides information about the primary sequence, chromosomal location, and expression of this mRNA in mutant cells. In the past year this work has been extended to include cloning and characterization of murine MCM, characterization of the MCM gene locus (MUT), and molecular cloning and analysis of mutant MCM cDNAs from MCM-deficient cells.

I. Characterization and Cloning of Murine MCM.

Murine MCM enzyme activity was assayed in primary murine fibroblasts and crude liver extracts and found to exhibit activity and reaction kinetics similar to human MCM. A full-length mouse MCM cDNA was cloned from a murine liver cDNA library, using the human MCM cDNA as a probe. The murine cDNA is 3,217 bases in length and encodes a protein of 748 amino acids (82,965 Da). There is 82% nucleic acid identity and 92% predicted amino acid sequence identity with human MCM. Extensive sequence divergence is apparent in the mitochondrial uptake sequence, with only 20/32 base identities.

The murine *Mut* locus was identified at chromosome 17C-D by *in situ* hybridization in cell lines containing a 2:17 Robertsonian translocation. This locus is syntenic with other loci on human chromosome 6 and murine chromosome 17, including the histocompatibility (HLA/H2) and glyoxalase (GLO1/Glo-1) loci. Linkage analysis among these loci performed in collaboration with Dr. Huda Zoghbi (human locus) and Dr. Jan Klein (mouse locus) indicates that although these loci are syntenic, they are not collinear, as a result of a series of rearrangements within these chromosomes subsequent to species divergence.

MCM expression has been studied by Northern blots of mRNA from various mouse tissues, including embryonic stem (ES) cells. MCM mRNA was found to be expressed ubiquitously (including ES cells) with levels highest in the liver and kidney. The level of expression roughly parallels the levels of assayable MCM and is consistent with the previously described "housekeeping" distribution of this enzyme.

II. Homology of Higher Eukaryote MCM with Prokaryote MCM.

A prokaryote MCM from *Propionibacterium shermanii* was cloned by Dr. Peter Leadlay. This MCM is a heterodimer of MUTA and MUTB subunits (human and mouse MCM are homodimers), and its function *in vivo* is to catalyze the reaction succinyl CoA→methylmalonyl CoA, which is the reverse of the reaction catalyzed by the human and mouse enzymes *in vivo*. Despite these structural and functional differences, homology is apparent among these sequences, with human MCM exhibiting 22% identity with MUTA and 65% identity with MUTB. No homology is apparent between the prokaryotic sequences and the mitochondrial targeting region of the eukaryotic protein.

III. Structure and Polymorphism Within the MUT Locus.

The human MUT locus has been cloned as a series of overlapping phage clones, restriction-mapped, and the exons and intron boundaries have been sequenced. There is a single gene encoding MCM that comprises 13 exons, spanning >50 kb. Consensus splice donor and acceptor sites were identified adjacent to each exon, and a GC-rich region is identified 5′ to the first exon consistent with the structure of a housekeeping promoter. A previously described *Hin*dIII polymorphism was localized within the coding sequence, where it does not affect the protein sequence. A method for detection of this polymorphism using the polymerase chain reaction has been developed and distributed to investigators interested in linkage or MMA pedigrees. Another polymorphism was identified, manifest as a 24 bp insertion/deletion in the open reading frame of the cDNA adjacent to the exon 6 boundary. This appears to be a rare but benign polymorphism. The genomic basis for this variant mRNA has not been elucidated. Additional sequence polymorphisms were identified at sites recognized by *Taq*I, *Xho*I, and *Nde*I. No discordance has been found between the segregation of mutant alleles and *Hin*dIII or *Taq*I polymorphic markers in a small number of families.

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IV. Characterization and Cloning of Mutant MCM.

MCM can result from genetic defects in the apoenzyme (termed *mut*) or genetic defects affecting synthesis of the adenosylcobalamin cofactor (*cbl*). A series of primary fibroblasts with *mut* MMA were identified, using an *in vitro* assay for the MCM apoenzyme. Further differentiation of mut^0 defects (which have no residual enzyme activity) from mut^- defects [which have residual enzyme activity often with abnormal K_m (adenosylcobalamin)] was made using an *in situ* assay. Southern blotting and Northern blotting were used to analyze *mut* cells. At least six distinct alleles are delineated by *Hin*dIII and *Taq*I haplotypes, the level of expression of MCM mRNA, and the biochemical phenotype. A statistically significant association was identified between the *Hin*dIII(–) polymorphism and alleles in mut^- cells. As described previously, several mut^0 cell lines have decreased mRNA, suggesting a primary defect in mRNA transcription or processing. A mut^0 cell line known to produce a small immunoreactive MCM protein that is not transported into mitochondria was found to have a termination mutation at codon 17. This mRNA produces antigenic material from an internal AUG distal to the mitochondrial targeting sequence. This mutation represents a complex prototype for a class of mutations in which absence of a targeting sequence leads to absence of a functioning gene product. Several distinct mutations have been found in mut^0 cell lines that alter amino acids preserved in alignments of human, mouse, and *P. shermanii* MCM sequences. Several sequence changes have also been identified in mut^- cells, although the effect of these sequence changes on enzyme activity has not yet been characterized. From these studies, Dr. Ledley and his colleagues hope to catalogue evolutionary sequence variations that preserve apoenzyme function and sequence changes that disrupt apoenzyme function, in order to highlight critical structural domains within the enzyme.

Dr. Ledley is also Associate Professor of Cell Biology and of Pediatrics at the Baylor College of Medicine.

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POST-TRANSCRIPTIONAL CONTROLS OF EUKARYOTIC GENE EXPRESSION

STEPHEN A. LIEBHABER, M.D., *Associate Investigator*

Dr. Liebhaber's laboratory studies the structure and function of eukaryotic genes, specifically the relationship of mRNA structure to its processing and function. These studies are carried out in two well-characterized model systems: the human α-globin gene family and the human growth hormone gene family.

I. Structural Basis for Splice-Site Selection.

Primary structural signals necessary for accurate splicing of RNA polymerase II transcripts are now defined in detail. It is clear, however, that these minimal signals, while necessary for splicing, are not sufficient and that additional signals—primary sequence as well as higher order structures—also appear to have a role in splice-site selection. The normal human pituitary growth hormone gene (hGH-N) consists of five exons. Two splice acceptor sites are used in intron 2, a major acceptor (B), used 90% of the time, and an alternative splice acceptor (B′) located 45 bases within exon 3 (B′), used ~10% of the time. The alternatively spliced mRNA encodes an hGH protein with a 15–amino acid internal deletion that may have physiologic functions distinct from the normal 22 kDa hGH. In contrast to this splicing pattern, the highly similar placental hGH-variant gene (hGH-V), fails to utilize the B′ acceptor site, despite an identical sequence surrounding both B and B′ splice acceptor sites. This laboratory is exploring the structural basis for this difference in the splicing patterns of these two highly related genes.

In an initial study, both hGH-N and hGH-V genes have been expressed in a variety of cell lines. Both genes maintain their respective splicing patterns in these lines, demonstrating that the splicing patterns are neither developmentally regulated nor tissue specific. Exon 3 and surrounding intron sequences were exchanged between these two genes, in order to map the determinants of the exon 3 splicing pattern. This exchange resulted in a parallel exchange in the splicing patterns. This suggested that exon 3 and/or adjacent intron 2 sequences contain essential determinants of splice-site selection.

To define these determinants more accurately, Dr. Liebhaber and his colleagues have introduced site-specific mutations within intron 2 and exon 3. Results demonstrate that the alternative splice is absolutely dependent on the presence of a single, specific adenosine (A) located 22 bases upstream of the B′ acceptor site. This A is present in hGH-N but not in hGH-V. Whether this base is the lariat branch A of the alternative splice acceptor is now being investigated. Additional mutations suggest that other sequences in the environment of the B and B′ splice sites can significantly alter the probability with which the alternative splice site is used. Studies now under way are focusing on the position and mode of action of these additional signals.

II. Relationship of mRNA Structure to Translational Efficiency.

Each mRNA species may have its own level of translational efficiency. Although the primary structural signals important in accurate translation initiation and termination have been known for some time, the structures that determine translational efficiency remain poorly defined. Dr. Liebhaber's laboratory is studying these signals in a well-characterized model system. The α- and β-globin genes encode the two protein subunits of hemoglobin. Equal quantities of α- and β-globin ($\alpha_2\beta_2$) are synthesized in the developing erythrocyte, despite a severalfold excess of α-globin mRNA. This balanced protein synthesis reflects a higher translational efficiency of β-globin mRNA. Initial experiments have demonstrated that β-globin mRNA is present on heavier polysomes than α-globin mRNA, despite their approximately equal sizes (145 and 141 codons, respectively). *In vitro* translation studies demonstrate that this difference in polysome loading reflects their relative abilities to load ribosomes during steady-state translation, as opposed to initial monosome assembly. Measurement of ribosome binding when the mRNAs are hybridized to cDNA fragments covering specific regions (hybrid arrest of translation initiation) demonstrated that the ribosome assembly site of α-globin mRNA requires more unimpeded space 3′ to the initiation codon than does β-globin mRNA to assemble an 80 S ribosome. This suggests a difference in the structure of the two respective ribosome assembly sites, which could impart a relative delay in ribosome loading of α-globin mRNA during steady-state translation. The 5′-nontranslated and 5′ proximal coding regions of these two mRNAs have been exchanged and chimeric α/β-globin mRNAs have been synthesized in order to map the regions responsible for this differ-

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ence. Preliminary studies suggest that certain of these exchanges result in an exchange in translational efficiencies. These investigations are being pursued to characterize further the structure(s) and mode(s) of action of the translational control determinant(s).

III. Interactions of mRNA Secondary Structure with the Translational System.

In previous studies this laboratory demonstrated that the elongating 80 S ribosome can melt out extensive duplexes formed between mRNA and cDNA. This may reflect the ability of the ribosome to unwind mRNA secondary structure during translation. This assumption is being tested directly. Site-specific secondary structures are being introduced into mRNAs, and their effects on translation are being monitored. Intramolecular duplexes are being positioned at specific regions within the mRNA to determine whether the proposed RNA helicase activity is present and whether it is dependent on active translation. The effects of the translational system on the stability of mRNA duplexes, and the reciprocal effect of such duplexes on translational efficiency, are being studied as critical components in the dynamic interactions that determine the compartmentalization, stability, and translational efficiencies of an mRNA.

IV. Expression and Function of the Human Growth Hormone Genes.

In collaboration with Dr. Nancy Cooke (University of Pennsylvania), this laboratory is also studying the expression and function of the human growth hormone genes. There are five functional genes in humans that encode the growth hormone–prolactin family of hormones: hGH-N; two placentally expressed chorionic somatomammotropin genes (hCS-A and hCS-B); hGH-V; and prolactin (hPRL). The encoded proteins are responsible for determining a number of functions critical to growth and reproduction.

Studies in this laboratory have defined the hGH-V gene as a placental growth hormone. This gene is expressed in the syncytiotrophoblastic epithelium of the villi, and the level of expression increases dramatically during gestation. Unlike the closely related pituitary growth hormone, hGH-V is expressed as an N-linked glycoprotein. Ongoing studies are attempting to define the function(s) of this protein by characterizing its pattern of receptor binding activity. The biological activities of the GH-PRL family of hormones are mediated by selective binding to two classes of membrane receptors, somatogen and lactogen. Primate growth hormones such as hGH-N are unusual, in that they bind to both classes of receptors. By using conditioned media from cells stably transformed with the hGH-V gene as a source of hGH-V, it has been possible to demonstrate that hGH-V is also able to bind to both receptor classes. Of additional interest, comparison of the relative binding affinities of hGH-N and hGH-V to somatogen and lactogen receptors demonstrates that hGH-V may be sevenfold more somatogenic than hGH-N. This finding, along with results from other laboratories, suggests that the hGH-V may be a gestational growth hormone of importance in the growth of the fetus and/or the metabolic regulation in the mother. Site-specific mutations within the hGH-V and hGH-N genes that alter a variety of the 13–amino acid differences between them should allow a more detailed understanding of the structural basis for their different receptor binding profiles.

Dr. Liebhaber is also Associate Professor of Human Genetics and Medicine at the University of Pennsylvania School of Medicine.

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FUNCTION AND REGULATION OF THE HEAT-SHOCK RESPONSE

SUSAN 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, the heat-shock proteins. This response is one of the most highly conserved genetic regulatory systems known. Dr. Lindquist's research is focused on 1) use of the response as a model system to investigate mechanisms of genetic regulation, particularly post-transcriptional regulation, 2) investigation of the functions of the heat-shock proteins in protecting cells from the toxic effects of stress and in normal growth and development, and 3) application of certain aspects of the response to other biological systems to solve a variety of practical problems.

I. Regulation of Heat-Shock Protein 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 in this organism is shifted from the production of normal cellular proteins to the production of heat-shock proteins. 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 is rapidly degraded during recovery. 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. To examine the mechanism of regulated hsp70 message degradation, Dr. Lindquist and her co-workers constructed and studied a variety of chimeric *HSP70* genes. It was determined that the 3′-untranslated region of the hsp70 message is sufficient to transfer regulated degradation to a heterologous message. The 3′-untranslated region shares sequence elements with unstable messages in other systems, such as the c-*myc* and c-*fos* messages in mammalian cells. Moreover, the c-*myc* message is stabilized by heat shock in *Drosophila* cells. Repression of hsp70 may be accomplished through a highly conserved mechanism employed by other cells in other circumstances.

II. Function of Heat-Shock Proteins.

Studies on the function of heat-shock proteins have focused on the yeast *Saccharomyces cerevisiae*, because of the ability to perform site-directed mutagenesis in this organism. Mutations that eliminate synthesis of hsp26 have no effect on the ability of cells to grow at high temperatures or to withstand short exposures to extreme temperatures. The *HSP83* gene exists in two copies. Disruption of either gene prevents cells from growing at high temperatures. Deletion of both genes is lethal. Thus this protein plays a vital role at all temperatures but is required by cells in higher concentrations for growth at higher temperatures. Cloning and sequencing of the *HSP35* gene demonstrated that it encodes glyceraldehyde-3-phosphate dehydrogenase. It may be induced at high temperatures to help restore normal ATP concentrations. Finally, and perhaps most importantly, hsp106 was found to be required for induced thermotolerance. Mutant hsp106 cells grow as well as wild-type cells at all temperatures and are killed at the same rate as wild-type cells when shifted directly from 25°C to 50°C. However, when wild-type cells are given a mild pretreatment at 37°C, they become tolerant to exposure to 50°C. The mutants do not.

III. Practical Applications of the Heat-Shock Response.

Another interest of the laboratory has been the development of a heat-inducible site-directed recombination system for *Drosophila*. Specifically, the site-specific flip (FLP) recombinase of the yeast 2μ plasmid and its recombination targets (FRTs) were transferred into the genome of *Drosophila melanogaster*. *Drosophila* were independently transformed, using P-element vectors, with two constructs: 1) an *FLP* gene under the control of *hsp70* regulatory sequences and 2) a *white* gene flanked by FRTs. When flies carrying both constructs were

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heat-shocked, synthesis of FLP recombinase was induced, catalyzing recombination between FRTs. This recombination caused loss of the *white* gene, seen somatically as white patches on a pigmented background in the eye. Less frequently it causes gain of a copy of the *white* gene, in tandem with the first, seen as patches of darker pigmentation. The frequency of loss and gain varies with the severity of the heat shock used to induce FLP synthesis. The patterns of somatic mosaicism produced by the heat shock are characteristic of the developmental stage at which it is applied. The recombinase is also active in the germline, producing white-eyed progeny carrying a single FRT (instead of *white* flanked by FRTs) and dark red-eyed progeny carrying two tandem copies of *white*. This system has a wide variety of potential applications for the genetic analysis of *Drosophila* and other organisms.

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*

The major goals of Dr. Lowe's research efforts are to understand both the mechanisms that regulate expression of cell surface oligosaccharide antigens and the functional consequences of these regulatory events. During the past year these efforts have focused on exploiting gene transfer systems developed in Dr. Lowe's laboratory for the isolation and molecular analysis of the glycosyltransferase genes that regulate expression of cell surface glycoconjugates.

Mammalian cells express a diverse array of oligosaccharide molecules on their surfaces. These molecules consist of carbohydrate structures covalently linked to membrane-associated proteins and lipids. Many of these cell surface oligosaccharide structures undergo striking changes during development and differentiation and in association with neoplastic transformation. A number of experimental observations suggest that cell surface oligosaccharides may function as information-bearing molecules that mediate communication between cells and their environment during development and differentiation. The structure of these cell surface oligosaccharides is determined primarily by glycosyltransferase enzymes. These enzymes act sequentially to synthesize the individual glycosidic bonds between the component monosaccharides within a final complex glycoconjugate. In general the oligosaccharide structures displayed by a tissue are thought to be determined by tissue-specific glycosyltransferase expression. However, the molecular mechanisms responsible for the regulation of expression of these enzymes, and thus the expression of cell surface glycoconjugate structure, are undefined. Moreover, in most instances the precise function(s) of the oligosaccharide structures determined by these enzymes is also obscure.

Dr. Lowe's laboratory is investigating representative human blood group glycosyltransferases as models to understand 1) the structure and regulation of these mammalian enzymes and 2) the function(s) of their oligosaccharide products during development and differentiation. These models include fucosyltransferases whose expression is determined by the human H and Lewis blood group loci. Each of these loci determines the expression of a distinct fucosyltransferase. The H enzyme constructs a fucosylated molecule, the H antigen, that serves as a precursor to the A and B blood group structures. By contrast, the Lewis enzyme constructs a fucosylated molecule, similar to but distinct from the H antigen, that is not directly involved in A or B blood group biosynthesis. These systems provide convenient models for understanding mammalian glycosyltransferase structure and regulation. The genetics of these systems are well understood yet informative; interesting alleles exist at each of 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.

Cloned glycosyltransferase genes represent tools to investigate these processes. However, cloning of glycosyltransferase genes has proven difficult, because these enzymes are typically present in minute quantities and are often unstable. Moreover, attempts to generate antisera against them have often yielded reagents that primarily recognize the highly antigenic oligosaccharide moieties of these glycoproteins. Thus conventional cloning schemes requiring these reagents have not been generally successful, except when the glycosyltransferase was abundant or the enzyme had been successfully purified several hundred thousand–fold. Cloning strategies based on gene transfer methods were developed to circumvent these difficulties; these strategies use existing information about the substrate and acceptor properties of these enzymes and take advantage of the multitude of antibody and lectin reagents specific for the surface-expressed oligosaccharide products of these enzymes. One of these approaches was used to isolate a human gene that encodes a fucosyltransferase with properties virtually identical to those of the blood group H fucosyltransferase. These studies have localized this gene to human chromosome 19, a finding consistent with linkage data that have assigned the H locus to this chromosome. Sequence analysis of the gene's cognate cDNA indicates that the enzyme is a type II membrane glycoprotein, consisting of a short, cytoplasmic amino-terminal tail, a single membrane-spanning segment, and a large carboxyl-terminal catalytic domain that resides within the Golgi lumen. Multiple and alternatively spliced transcripts are generated from this gene in some cell lines, suggesting diverse possibili-

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ties for control of this gene's expression. Structural and functional analysis of these transcripts is in progress.

A gene transfer approach was also used to isolate a human cDNA that encodes a fucosyltransferase different from the H fucosyltransferase. Analysis of the catalytic properties of this enzyme indicates that it has the unique ability to generate two distinct glycosidic linkages. The substrate properties of this enzyme are virtually identical to those exhibited by the Lewis blood group fucosyltransferase. The cognate gene was localized to human chromosome 19, consistent with genetic linkage data for the Lewis locus. The sequence of this cDNA indicates that it also encodes a type II transmembrane protein. Surprisingly, despite the fact that the H and Lewis enzymes maintain identical structural topologies and have nearly identical substrate requirements, no significant primary sequence similarities can be found between them. One oligosaccharide product of this enzyme represents a murine stage-specific embryonic antigen. Studies are in progress to define the developmental expression patterns of this gene, in preparation for transgenic animal experiments designed to explore the function(s) of the cell surface molecules it determines.

A murine galactosyltransferase cDNA has also been isolated by Dr. Lowe's laboratory, using gene transfer methods. This enzyme is also a type II transmembrane molecule but bears no primary structural similarity to any other glycosyltransferase. Biochemical analyses using cell lines transfected with this gene have confirmed that the complement of oligosaccharide structures displayed on the cell surface reflects the kinds and specific activities of the cognate glycosyltransferases within a cell. These studies also indicate that the dynamic changes in cell surface oligosaccharide structures that accompany development are associated with significant changes in glycosyltransferase gene expression. Studies are in progress to define the expression patterns of this gene during development, again in preparation for transgenic experiments designed to answer questions about the function(s) of cell surface oligosaccharide molecules during mammalian development.

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

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HUMAN RETROVIRUSES AND COORDINATE GENE EXPRESSION IN T CELLS

GARY J. NABEL, M.D., PH.D., *Assistant Investigator*

A major interest of Dr. Nabel's laboratory is to understand how viruses interact with host cell factors to replicate successfully in eukaryotic cells. Viral replication is often dependent on transcription factors relevant to cell proliferation and differentiation. These studies therefore not only address mechanisms of viral gene regulation but also provide insights into eukaryotic gene expression.

Dr. Nabel previously showed that activation of T cells, which increased human immunodeficiency virus (HIV) expression up to 50-fold, correlated with the induction of a DNA-binding protein, NF-κB. The laboratory has begun the identification of cellular genes regulated by NF-κB, to understand the mechanism of transcriptional activation by NF-κB, to determine its role in HIV activation in other cell types (e.g., monocytes), and to identify genes encoding κB binding proteins. The cellular genes regulated by NF-κB in non–B cells were previously unknown, although related sites are present in the enhancers of several genes, including class I and II major histocompatibility genes and β_2-microglobulin. A sequence related to the κB upstream region of the interleukin-2 receptor α (IL-2Rα) gene, designated IL-2R κB, has been identified. It is identical to κB at 9 of 11 base pairs and competes for binding to NF-κB. Unlike κB in Ig light chain or HIV, mutation of this site does not affect induction by phorbol myristate acetate (PMA); however, this site is required for stimulation of the IL-2Rα enhancer by tax_1 of human T cell leukemia virus I (HTLV-I). These data suggested that a κB-like site may play an important role in the regulation of a T cell–specific gene and that there is heterogeneity in function that may be mediated by related κB sites.

To understand potential mechanisms of HIV activation, Dr. Nabel has examined viral transactivators that stimulate the HIV enhancer. Viral genes may provide insight into how HIV expression could be activated and may also be clinically relevant to cofactors that accelerate HIV disease. Cotransfection of the adenovirus E1A gene had previously been shown to increase HIV-CAT (chloramphenicol acetyltransferase) expression in Jurkat cells, and activation by E1A was dependent on the TATA box. Because E1A stimulated the HIV enhancer through the TATA box, the possibility was considered that the TATA box mediated activation by NF-κB in this enhancer. When HIV-CAT plasmids with mutant TATA boxes were transfected into Jurkat cells, they remained responsive to PMA activation through NF-κB and used the same transcriptional initiation site, even though basal levels of expression were reduced 10- to 50-fold. Inversion of the TATA box or substitution with the SV40 TATTT sequence revealed a similar response, suggesting that the TATA sequence in the HIV enhancer functions as a position- and orientation-dependent, positive regulatory element.

Although cells of the monocyte lineage provide a reservoir for HIV production *in vivo*, the regulation of HIV transcription in these cells had not been defined. When regulatory elements required for HIV gene expression in the monocyte lineage were examined, Dr. Nabel and his colleagues found that it was regulated by NF-κB; however, the regulation of NF-κB in the monocyte lineage differed from T cells. NF-κB binding became active at a discrete stage of monocyte differentiation and was thereafter present constitutively in the mature monocyte and macrophage. Its binding activity correlated with the transition from promonocyte to monocyte. In a chronically infected promonocytic cell, U1, HIV-1 replication was also activated by cellular differentiation, and this activation correlated with NF-κB binding activity. These findings suggested that NF-κB binding and subsequent HIV expression are associated with differentiation in the monocyte lineage. Other factors, such as granulocyte macrophage colony-stimulating factor (GM-CSF), apparently exert their control through a mechanism independent of NF-κB, since GM-CSF does not induce NF-κB binding, and activate HIV expression in mature macrophages, which already contain NF-κB. These findings suggest that at least two signals modulate synthesis and secretion of HIV in mononuclear cells, with NF-κB induction in the monocytic lineage providing one signal that may initiate viral transcription.

NF-κB binds to the IL-2R κB site, but the IL-2R κB site differs from κB because it does not respond to induction by PMA in Jurkat T leukemic cells, suggesting possible heterogeneity among proteins that bind to related κB-like sites. To define the biochemical basis of κB specificity, the laboratory has developed binding conditions for the electrophoretic mobility shift assay to detect other κB binding proteins. This approach was used to define an IL-2R κB binding protein distinct from NF-κB. Binding of this factor to the IL-2R κB site under these conditions is

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not competed by κB or negative control competitors in molar excess. By UV crosslinking, protein complexes of ~160, ~120, and ~95 kDa that interact with this site have been detected. DNA footprinting suggests that this protein contacts DNA both within the IL-2R κB sequence and in the flanking region.

Further evidence was recently obtained for the existence of such a protein by identifying a cDNA clone that binds specifically to the IL-2R κB sequence. By Northern blot analysis, this clone hybridizes to a cellular mRNA ~5 kb in length. It is expressed in lymphoid cells and exists in a single copy in the genome. This gene differs from a previously described cDNA that binds to the κB and H2TF1 sites, suggesting that there is heterogeneity among the genes and proteins that bind to κB-like sites.

Dr. Nabel has begun to develop systems that utilize viral vectors to express biologically active proteins in cells and tissues *in vivo*. A method to express recombinant genes within endothelial cells of the arterial wall of pigs, using a murine amphotropic transducing retroviral vector in endothelial cells, has been developed. Although β-galactosidase was used for initial studies, other biologically active proteins, including angiogenic factors, growth factors, or transcription regulatory proteins, are being introduced. The goal of this research is to understand basic mechanisms of gene regulation and transcriptional activation and to define the biological significance of factors that regulate gene expression in complex organisms.

Dr. Nabel is Assistant Professor of Internal Medicine and Biological Chemistry and a member of the Cell and Molecular Biology Department at the University of Michigan Medical School.

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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 laboratory has continued to focus on three main areas of research: 1) developmental biology of the contractile system, with emphasis on skeletal and cardiac myogenesis; 2) the mechanisms involved in the production of multiple protein isoforms from a single gene by alternative splicing; and 3) structural and functional characterization of genes involved in the regulation of the contractile phenotype.

I. Developmental Regulation of the Contractile System.

One characteristic of the terminally differentiated phenotype of striated muscle cells is its apparent irreversible withdrawal from the cell cycle. Although these cells have functional receptors for a variety of growth factors, as demonstrated by their metabolic response, they are not induced to re-initiate DNA synthesis. It is not known whether these cells have lost their ability to replicate their DNA or whether their lack of response is due to the presence of a repressor of cell growth that is induced during differentiation. The previous isolation of cells that are temperature sensitive for the commitment step and cannot withdraw irreversibly from the cell cycle, together with the demonstration that the BC3H1 cell line is a mutant for this phenotype, strongly suggests that an inhibitor of cell growth is induced during terminal differentiation. To develop an experimental approach to test this hypothesis and identify the putative molecule(s) involved, Dr. Nadal-Ginard and his colleagues have explored the ability of the SV40 large T antigen to inhibit myogenesis. The temperature-sensitive mutant tsA58, driven by the metallothioneine promoter, has been used to show that expression of this oncogene for as little as 5 h in terminally differentiated muscle cells is sufficient to make the cells respond to growth factors and reenter the cell cycle. The multiple nuclei in these myotubes undergo synchronous mitosis and, in some cases, cytokinesis. A similar phenomenon is induced by the adeno-E1A protein as well as the phyloma T antigen. These results strongly suggest that the terminally differentiated phenotype is maintained by a gene product whose function is neutralized by the oncogene tested. Preliminary results indicate that the retinoblastoma gene product is not responsible for this phenotype. The involvement of p53 in this process is being investigated. In addition, a large variety of SV40 T antigen mutants that are defective in their interaction with different cellular components are being studied, in order to map the domain of this protein responsible for the reversibility of the terminally differentiated phenotype. Experiments designed to identify the molecule that putatively interacts with SV40 T antigen through immunoprecipitation of T antigen under different conditions are also under way.

One of the main obstacles in cardiac biology has been the unavailability of cardiocyte cell lines. To test the generality of the effect of SV40 T antigen on terminally differentiated phenotypes, Dr. Nadal-Ginard and his colleagues have transfected primary cardiocytes with the tsA58 mutant under the control of cardiac-specific promoters. A large number of established cell lines have been produced. These cells have many of the characteristics of very early cardiocytes and depend on the expression of T antigen for growth. At permissive temperatures these cells respond with rapid cell growth to serum growth factors and remain undifferentiated. At nonpermissive temperatures the cells become unresponsive to growth factors and induce a large array of genes characteristic of the differentiated state. Approaches similar to those described for the skeletal muscle cells are in progress to identify the molecule(s) interacting with T antigen and responsible for the withdrawal from the cell cycle.

The transcriptional induction of contractile protein genes continues to be studied, using the myosin heavy-chain gene as a model system. Several cis-acting elements on these promoters have been identified. None of them is tissue specific. Recently the transacting factor that binds to the light-chain enhancer has been identified and characterized. Experiments are in progress for the purification and eventual cloning of this molecule. At the same time the role of different isoforms of the thyroid hormone receptor on the induction and repression of different myosin heavy-chain genes continues to be pursued.

II. Alternative Splicing of Contractile Protein Genes.

The laboratory has continued to analyze the regulation of alternative splicing, with emphasis on mutually exclusive exons, which seem the most likely to reveal the underlying molecular mechanisms. Two main questions have been addressed: What is the basis of this mutual exclusivity? How is

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exon selection regulated? Minigene constructs containing different arrangements of the α-tropomyosin (α-TM) exons 2 and 3 have been used for *in vivo* and *in vitro* analyses. The mutual exclusivity is based on the peculiar anatomy of the intron between these two exons. The branch point sequence is located very distantly from the acceptor site and too close to the donor site. This proximity creates steric hindrance between the splicing factor bound to the 5′ splice site and the branch point that prevents a productive interaction. When this distance is increased, the mutually exclusive behavior is eliminated. These experiments have been explored to redefine the characteristics of the 3′ splice site of the intron. The results indicate that the branch point of an intron is defined by a constrained sequence and its close association with a polypyrimidine tract. The 3′ splice site, on the contrary, does not require a polypyrimidine and is defined as the first AG downstream of the branch point. The choice between mutually exclusive splice sites, at least in the α-TM gene, is governed by the strength of the polypyrimidine tract. Swapped experiments *in vivo* and *in vitro* have demonstrated that it is possible to change the pattern of exon usage in a predictable manner. The behavior of different polypyrimidine tracts in splicing correlates with their affinity for the splicing factor U2AF, as demonstrated by UV crosslinking experiments.

III. Analysis of Genes Involved in the Regulation of the Contractile State.

To address some of the unresolved aspects of excitation-contraction coupling, the laboratory has cloned from skeletal and cardiac muscle two of the main genes involved in the process, the α-subunit of the dihydropyridine-sensitive calcium channel and the calcium release channel of the sarcoplasmic reticulum. Expression vectors with the wild-type and *in vitro* mutagenized sequences are being tested, in order to initiate a systematic analysis of structure-function relationships in these molecules. At the same time the kinetic properties of a cloned skeletal muscle potassium channel that is the homologue of the Shaker channel in *Drosophila melanogaster* have been analyzed in mammalian cells and in frog oocytes. From the kinetic properties a functional model of the channel that is experimentally testable has been developed. A systematic mutation of the S4 region of this channel is under way, to test the model and elucidate the basis for the voltage sensitivity of this molecule. These experiments are being carried out in collaboration with Dr. Peter Hess (Harvard Medical School).

Dr. Nadal-Ginard is also Professor of Pediatrics and of Cellular and Molecular Physiology at Harvard Medical School.

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GENOMIC RESPONSE TO GROWTH FACTORS

DANIEL NATHANS, M.D., *Senior Investigator*

Extracellular signaling agents—hormones, growth and differentiation factors, neuromodulators, and depolarizing agents—act via second messenger systems to induce genetic programs in target cells. Research in Dr. Nathans' laboratory is aimed at characterizing genes that are part of the program induced by growth factors and defining the function of their encoded proteins. By screening mouse 3T3 cell cDNA libraries for clones derived from mRNA present in cells stimulated by serum growth factors but not in unstimulated cells, Dr. Nathans and his colleagues have identified a set of genes that is activated coordinately with the proto-oncogene c-*fos* or c-*myc* during the first several minutes after addition of serum, platelet-derived growth factor, or fibroblast growth factor; other genes that are activated later have also been identified. Several of the "immediate early" genes encode known or probable transcription factors that are thought to regulate the genetic program induced by growth factors. These genes and the proteins they encode have been the main focus of the laboratory's research during the past year.

I. A New Member of the Steroid and Thyroid Hormone Receptor Superfamily.

One of the cDNA clones (nur77) derived from an immediate early mRNA was found to encode a protein of 601 amino acids homologous to steroid and thyroid hormone receptors. The Nur77 protein contains two regions of sequence similarity to the hormone receptors, corresponding to their DNA-binding and ligand-binding domains. In each case the degree of similarity resembles that found between other members of the family. A homologous mRNA was detected by Dr. Jeffrey Milbrandt in rat pheochromocytoma cells after stimulation by nerve growth factor. Studies of Nur77 are continuing in the laboratory of Dr. Lester Lau.

II. Characterization of the Zinc Finger Protein Zif268.

As reported earlier, the *zif268* gene encodes a protein of 533 amino acids with three tandemly repeated, typical zinc finger sequences. (The cDNA has been isolated in three other laboratories and designated NGFI-A, erg-1, and Krox24.) Upstream of the gene are potential binding sites for known transcription factors: AP-2, AP-1, cAMP-binding protein, and four serum response element (SRE) core sequences [CC(A or T)$_6$GG], known to be part of a signal involved in the activation of the c-*fos* gene by serum growth factors. To determine whether the core SREs upstream of the *zif268* gene mediate induction, various deletion constructs of the *zif268* promoter region and synthetic oligonucleotides corresponding to each of the four *zif268* putative SREs were tested for responsiveness to serum, platelet-derived growth factor, and phorbol ester. Each of the SREs conferred inducibility to these agents, and multiple SREs resulted in greater inducibility than a single element. Each of the *zif268* SREs also competed with the c-*fos* SRE for binding by serum response factor present in HeLa cell nuclear extract, even though the sequences that flank the core SRE sequences are different. Thus the *zif268* SREs are functional and probably account for the coordinate induction of the *zif268* and c-*fos* genes.

Based on its three zinc finger sequences, Zif268 is thought to be a sequence-specific DNA-binding protein that regulates transcription of genes that are part of the growth factor–induced genetic program. To begin a search for genes that are regulated by Zif268, DNA sequences to which Zif268 binds have been identified. On the assumption that the *zif268* gene itself has a binding site in its promoter region, fragments of DNA derived from the upstream region of the gene were incubated with Zif268 produced in *Escherichia coli*, and binding was assessed by electrophoretic separation of bound and unbound fragments. Two binding sites were found and confirmed by DNase I footprinting. Similar sequences are present in the promoter regions of a number of other genes, including other immediate early genes. Several were shown to be Zif268-binding sites. The consensus binding site sequence, inferred from natural and synthetic sites, is GCG $^{G}_{T}$GGGCG.

III. Interaction of Jun and Fos.

Two immediate early genes encode proteins related to the oncoprotein v-Jun and the transcription factor AP-1. One of these is the proto-oncogene product c-Jun, and the other is Jun-B. A third member of the *jun* gene family (*jun-D*) was isolated by screening murine genomic and cDNA libraries. It is expressed in nonproliferating cells and is only slightly induced by serum growth factors. The three

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Jun proteins are very similar in their basic DNA-binding regions, in their leucine zipper sequences, and in the putative transcription-activation segments. Each of the proteins forms homodimers (or heterodimers with another Jun protein) and binds weakly to an AP-1 site or cAMP response element (CRE) in DNA. However, in the presence of Fos or Fra-1 the binding affinity is greatly enhanced. Thus Jun/Fos or Jun/Fra-1 heterodimers are the most active binding species, and the DNA-binding site specificity of the various Jun/Fos or Jun/Fra-1 heterodimers is very similar.

The current model for the binding of Fos/Jun heterodimers to an AP-1 site or CRE pictures the monomers held together by a coiled coil structure involving their leucine repeat helices, with their basic regions contacting symmetrical halves of the binding site. To test the prediction that in the Fos/Jun heterodimer the basic region of Fos confers specific DNA-binding properties equivalent to those of the basic region of Jun, truncated Fos/Jun chimeric proteins were prepared, consisting of the basic region of one protein joined to the leucine repeat of the other. Heterodimers with mixed Fos and Jun leucine repeat segments showed high-affinity binding to the AP-1 site or CRE, whether they contained two basic regions from Jun, two basic regions from Fos, or one from each source. Heterodimers with two Fos basic regions showed somewhat greater affinity for the CRE and AP-1 site than the heterodimer with two Jun basic regions. The DNA sequence specificity and the purine and phosphate DNA contact sites for each heterodimer were similar. Thus in the Fos-Jun heterodimer the basic region of Fos contributes specific DNA-binding properties equivalent to those of Jun. These results support a model in which the Fos and Jun basic regions of the Fos-Jun heterodimer each interact with symmetrical DNA half-sites; the differences in DNA binding of Fos + Jun versus Jun or Fos alone are due to the variable dimerization properties of Fos and Jun.

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., *Assistant Investigator*

Visual pigments are the light-absorbing proteins in the retina that mediate the first step in visual excitation. They are members of a large family of cell surface receptors that transduce external stimuli by activation of G proteins.

One area of interest in Dr. Nathans' laboratory is the chemistry of visual pigment photoactivation. Each pigment consists of a chromophore, 11-cis retinal, joined covalently to an integral membrane protein, opsin. Photon absorption leads to isomerization of the chromophore to an all-trans configuration. This isomerization drives a series of conformational changes in the attached opsin. These changes can be followed spectroscopically, and the intermediates in photoactivation can be trapped at low temperature. As a first step in defining the 11-cis retinal-binding pocket and characterizing its electronic environment, Dr. Nathan and his co-workers have studied a series of site-directed mutants carrying alterations in the putative retinal-binding pocket of bovine rhodopsin. Fourteen mutants were constructed that replaced charged amino acids predicted to contact retinal. These alterations were chosen because retinal is known to undergo a significant change in dipole moment upon photoexcitation and therefore should be sensitive to electronic perturbations. The expectation was that these mutants would combine with 11-cis retinal to form visual pigments with novel absorbance spectra. Instead the experiment shows that each has a nearly normal spectrum. These results suggest that either the current binding pocket model is incorrect or the commonly held hypothesis of retinal tuning by nearby negatively charged residues is incorrect. To test the latter possibility, which is thought to be the most likely, 15 site-directed mutants have been constructed, in which each of the remaining negatively charged amino acids in bovine rhodopsin has been replaced by a neutral amino acid.

A second area of interest is the study of inherited variations in human vision. In one set of experiments, Dr. Charles Weitz has initiated a study of inherited variation in blue sensitivity. Affected persons in two families from Japan show the same single–amino acid change in their blue pigment genes, resulting in a glycine-to-arginine change in the putative second transmembrane segment. Linkage analysis, together with expression and characterization of the mutant protein, will be required to test definitively whether this change causes the mutant phenotype. In another set of experiments the genetic alterations responsible for a rare X-linked trait, blue cone monochromacy (also called incomplete achromatopsia), have been determined. Affected individuals show 1) nonfunctional red and green cone systems, leading to a complete absence of color sense; 2) photophobia; 3) low acuity, typically 20/80 to 20/200; 4) nystagmus; and 5) in some families a slowly progressive macular scarring. Fourteen families have been analyzed: ten carry deletions in the red and green pigment gene cluster that range in size from 0.6 to 54 kb; four have lost all but one of their red and green pigment genes, and sequence analysis shows that the remaining gene has suffered a point mutation. The deleted regions all remove a common 0.6 kb segment that lies ~4 kb upstream of the red and green pigment gene cluster. This region may contain a long-range enhancer analogous to the one defined by Grosveld and his colleagues adjacent to the β-globin gene cluster. Transgenic mouse experiments in which this region is joined to a reporter gene are in progress to test this hypothesis.

Dr. Nathans is also Assistant 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 determination of cellular phenotype is a function of the regulated expression of a set of unique genes under a particular set of circumstances. Therefore an understanding of the molecular mechanisms that control gene expression is central to an understanding of the complex cellular events that take place during such events as embryogenesis and oncogenesis. Dr. Nevins's laboratory is focused on the elucidation of the molecular events that control gene expression, beginning with the identification and isolation of factors that directly interact with the gene or primary transcripts of the gene.

I. Trans-Activation of Transcription.

Complex cellular events are often best studied through the use of simple model systems. The control of transcription mediated by the adenovirus gene product has served as a useful means to understand the workings and control of transcription factor activity in eukaryotic cells. Dr. Nevins is attempting to elucidate the underlying mechanisms of trans-activation brought about by viral regulatory genes such as E1A. The early adenovirus E2 gene, a viral transcription unit, has been used to define the mechanism of E1A action. Previous experiments demonstrated that adenovirus infection of human cells leads to an E1A-dependent activation of the DNA-binding capacity of a cellular transcription factor, E2F, that binds to two sites in the viral E2 early promoter. These E2F recognition sequences have been shown to be critical for E1A-dependent E2 transcription, and the E2F-binding sites can confer E1A-induced transcription to a heterologous promoter. In addition, under a variety of circumstances the increase in E2F-binding activity coincides with the activation of E2 transcription. Recent experiments have demonstrated that in addition to the E1A gene, the early viral E4 gene is also necessary for the activation of E2F-binding activity. Measurements of E2 RNA production in cells infected with an E4 mutant, as well as various transfection assays, demonstrate that the E4 gene can trans-activate the E2 promoter and that the E4 gene is essential for full E2 transcription in addition to E1A. A further analysis of the activation of the E2F factor has yielded information concerning the respective roles of the E1A and E4 gene products.

These experiments demonstrate that the enhanced binding of E2F to the E2 promoter is the result of two separate events. First, there is a stimulation of the DNA-binding activity of the E2F factor, and this stimulation is E1A-dependent but independent of the E4 gene. Second, there is an induction of a stabilized interaction between two E2F molecules bound to adjacent promoter sites. An E2F factor bound to a single site rapidly dissociates from the DNA, whereas the dissociation from a double-site interaction is much slower. This is only true, however, for the E2F factor isolated from infected cells. The low level of E2F from mock-infected cells shows no evidence of this stable binding, even at high concentrations, where two E2F factors can be driven onto the promoter. The induction of this stable binding capacity requires the E4 gene. Thus the activation of E2F during an adenovirus infection is a two-step process involving a change in both the DNA-binding activity of the factor and the capacity to stabilize the interaction through protein-protein contacts. This latter function is of particular interest because of the potential importance of combinatorial interactions of transcription factors with promoter elements.

Cell-free extracts have been used to develop an assay for the *in vitro* activation of the DNA-binding activity of E2F. E2F activity is undetectable in extracts of HeLa cells, but upon incubation with a fractionated extract from adenovirus-infected cells there is an ATP-dependent increase in E2F DNA-binding activity. This increase does not occur when an equivalent fraction from E1A mutant-infected cells or E4 mutant-infected cells is used. Incubation of E2F with phosphatase inactivates the E2F DNA-binding activity. Incubation of the phosphatase-inactivated E2F with an infected cell fraction restores E2F activity, as does incubation with a known protein kinase. In contrast, incubation with an extract from mock-infected cells does not restore activity, thus indicating that at least part of the activation of the DNA-binding activity of E2F is regulated by phosphorylation.

Finally, it has been found that the normal cellular role of E2F may involve participation in a signal transduction pathway relating to cell proliferation. E2F binds to two sequence elements within the P2 promoter of the human c-*myc* proto-oncogene that are within a region that is critical for *myc* promoter activity. Furthermore, analysis of the gene sequence library for potential E2F-binding sites identified a

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number of genes that contained sequences similar to the E2F recognition site. These include genes such as the c-*myb* proto-oncogene, the N-*myc* proto-oncogene, the DHFR gene, and the EGF receptor gene. These genes are commonly regulated during cell proliferation, suggesting a role for E2F in this process. Indeed, serum-stimulated expression of the c-*myc* promoter in 3T3 cells is dependent on the E2F sites. Furthermore, the DNA-binding activity of E2F is increased fourfold upon serum stimulation, and this increase is independent of new protein synthesis, as is the stimulation of c-*myc* transcription upon serum stimulation.

II. Control of RNA Processing.

In addition to the control of transcription initiation, it is now clear that post-transcriptional control of gene expression can be an important mechanism of gene regulation. Often a transcription unit encodes not one mRNA but several mRNAs. Through alternative processing of the primary transcript, one particular mRNA, and thus one particular protein, is selected and produced. In several cases this alternative processing has been shown to be a regulated event subject to change, depending on the circumstances in the cell. Alternative processing can involve both the selective splicing of exons and the selection of one of several poly(A) addition sites, thus generating the mRNA 3′ terminus. Possibly the best example of regulation of poly(A) site utilization is within the differentiating B lymphocyte, where there is a dramatic change in the nature of immunoglobulin heavy-chain polypeptides that are synthesized. The RNAs that direct the synthesis of the two forms of heavy-chain proteins are encoded in the same transcription unit and are produced by the differential cleavage of the primary transcript at two different poly(A) sites. A definition of the events and factors involved in poly(A) site formation using transfection assays and *in vitro* systems has been a major goal of the laboratory.

Recently efforts have been directed at the analysis of cell-free systems that carry out poly(A) site processing *in vitro* to isolate factors involved in this reaction and define their role in the processing event. Four HeLa cell nuclear factors that are required for specific pre-mRNA cleavage and polyadenylation have now been extensively purified. Two factors, PF1 and PF2, are required for specific polyadenylation of the cleaved RNA. PF1 is a poly(A) polymerase, and PF2 is a factor that confers specificity to the reaction by allowing recognition of the AAUAAA sequence, an element that is essential for poly(A) site utilization. Both of these factors, along with two additional factors, CF1 and CF2, are required for the endonucleolytic cleavage of the pre-mRNA. The ability of each of these factors to form specific complexes with the pre-mRNA has been assayed using native gel electrophoresis. Two distinct complexes were detected. PF2, the specificity factor, forms an initial complex with the pre-mRNA dependent on the AAUAAA sequence element but independent of specific downstream sequences. Formation of the PF2 RNA complex permits the subsequent interaction of CF1 and the formation of a second larger complex. CF1 binding requires the downstream sequence element in addition to PF2 binding. Whereas the PF2 RNA complex is unstable and rapidly dissociates, the ternary complex formed by a CF1/PF2 and the RNA is stable. Thus the interaction of CF1 dependent on the downstream sequence element can be viewed as a commitment of the poly(A) site for processing. Upon the addition of the poly(A) polymerase (PF1) and the CF2 factor, the pre-mRNA is specifically cleaved at the poly(A) site and then polyadenylated. With these factors in hand and distinct assays for each factor available, the possibility exists of assaying a regulated system such as the differentiating lymphocyte to define which factor is the rate-limiting component in poly(A) site utilization.

Dr. Nevins is also Professor of Microbiology and Immunology at the 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 extended its research efforts in mapping and characterizing a number of human X-linked diseases.

Mental retardation with Xq27 fragile site has remained a major focus in the laboratory. Understanding the nature of the fragile site requires isolation of DNA from the region of the mutation. Although new yeast vectors for yeast artificial chromosomes were originally used, an apparently high rate of recombination of artificial chromosomes made with these vectors convinced this laboratory to return to the original vectors of Burke and Olson. A new library is now being constructed from a hybrid containing a translocation between a host rodent chromosome and a human X, with the human breakpoint at what is felt to be the Xq27 fragile site. Dr. Stephen Warren, a collaborator, made this hybrid by inducing the fragile site in a hybrid containing an intact human X and then screening for breakage at the fragile site and loss of the distal Xq28 markers. The immediate goal is to identify an artificial chromosome containing the translocation breakpoint, in order to obtain human sequences from in and around the fragile site.

Continued progress has been made in the study of the X-linked retinal dystrophy choroideremia. A female with choroideremia due to an X;13 translocation, with nonrandom inactivation of the normal X, allows more precise localization of the disease locus. Using the marker DXS165 (identified by Dr. Ropers in Holland as being within deletions in patients with choroideremia) the laboratory has initiated jumping and walking experiments that have allowed isolation of sequences within 40 kb of the translocation breakpoint. These sequences are being used for continued walking experiments and are being screened for exons of genes expressed in the retina and retinal pigmented epithelium in a search for a candidate gene for this disease.

The Lowe oculocerebrorenal syndrome is an X-linked disorder characterized by mental retardation, renal tubular abnormalities, and congenital cataracts. In collaboration with Richard Lewis of the Cullen Eye Institute at Baylor College of Medicine, X-linked restriction fragment length polymorphisms (RFLPs) have been used to study six families in which the Lowe syndrome is segregating. Two markers mapping to the region Xq24–26 were identified that are tightly linked to the disease locus. One locus, DXS42, shows no recombinants with lod score of 8.67; the other, DXS10, is linked at 2% recombination with lod score 8.89. However, DXS10 and another RFLP, DXS86, share large restriction fragments on field-inversion gel electrophoresis analysis, and DXS86 is linked at 10% with lod 4.74. This discrepant result occurs because one family in which DXS86 is informative and shows crossovers is not informative for DXS10.

Fibroblasts from a female affected with Lowe syndrome due to an X;3 translocation were used to construct somatic cell hybrids in which the derivative 3 containing only Xq25-qter and the derivative X containing Xpter-q25 have been isolated from the normal X. With such hybrids, DXS10 and DXS86 are shown to be distal to the breakpoint, while DXS42 and DXS37 are proximal.

Dr. Nussbaum is also Associate Professor of Human Genetics and Pediatrics at the University of Pennsylvania School of Medicine and Attending Physician in Genetics and Metabolism at Children's Hospital of Philadelphia.

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GENETIC HEMATOLOGY

STUART H. ORKIN, M.D., *Investigator*

The central focus of Dr. Orkin's laboratory is the study of the structure, expression, and function of specific genes that are relevant to the normal biology and pathology of hematopoietic cells. The ultimate goals are to understand the molecular basis for cellular commitment and differentiation, determine the mechanisms for regulation and coordination of gene expression in developing blood cells, and apply basic knowledge to the management of inherited human disorders of hematopoietic cells.

I. Globin Gene Expression.

The genes of the β-globin family are subject to precise regulation, both with respect to cell lineage and the temporal expression of individual members. To approach molecular mechanisms of gene regulation, Dr. Orkin initially directed attention to the identification of nuclear DNA-binding proteins that might participate in control of fetal (γ)-globin gene transcription. An erythroid cell-specific factor (GF-1) was detected that recognizes a short-sequence motif present in the γ-globin promoter in the vicinity of a single-base change (at position −175) associated with hereditary persistence of fetal hemoglobin (HPFH) into adult life. By expression of site-specific mutagenesis it was established that the overexpression of this mutant promoter is erythroid-specific and is dependent on the availability of binding sites for the erythroid factor. Independent work by others showed that the sequence motif recognized by this erythroid factor is not limited to the γ-globin promoter but is found in the promoters and enhancers of the majority of erythroid-expressed genes of human, mouse, and avian origin.

The human protein was purified to homogeneity and subjected to direct peptide sequencing to determine the nature of the erythroid factor. In addition, the cDNA encoding the mouse protein was isolated and characterized by expression of recombinant clones in mammalian cells. GF-1 of both human and mouse origin is a 413–amino acid polypeptide that contains two novel cysteine-cysteine zinc finger–like DNA-binding domains. This DNA-binding region is extraordinarily conserved across species. GF-1 is expressed not only in developing erythroid cells but also in cells of the megakaryocytic (platelet) lineage. This indicates that this regulatory factor is expressed in a bipotential hematopoietic progenitor that subsequently further commits to a single-cell lineage. GF-1 is encoded by a single gene on the X chromosome. Current efforts are devoted to understanding the role of GF-1 in transcriptional activation of genes, the regulation of the GF-1 gene in developing hematopoietic cells, and the involvement of GF-1 in cellular commitment and hemoglobin switching in development.

II. Molecular Genetics of Superoxide Generation by Phagocytes.

Phagocytic cells produce superoxide via an NADPH-oxidase complex as part of a major bactericidal host defense system. Deficiency in this system leads to an immune disorder, chronic granulomatous disease (CGD), the major variety of which is X-linked (X-CGD). By reverse genetics it was previously established that X-CGD is due to mutation of a gene encoding the glycoprotein subunit of an unusual, neutrophil-specific heterodimeric cytoprotein *b*. Subsequent research has proceeded in three directions. 1) Mutations leading to X-CGD and rarer autosomal forms of CGD have been characterized in an effort to delineate critical portions of the relevant proteins. These studies have led to the identification of a single-base change in the *X-CGD* gene that results in a nonfunctional cytochrome *b* molecule in white blood cells and to the discovery of mutations in the other subunit of the cytochrome in a rare form of autosomal CGD. 2) The molecular basis for regulation of the *X-CGD* gene in white blood cells has been pursued by expression of gene constructs and study of nuclear DNA-binding proteins that interact with promoter elements. 3) The mechanism(s) by which the cytokine interferon-γ augments transcription of the *X-CGD* gene and ameliorates the cytochrome *b* deficit in some X-CGD patients has been investigated. These studies have revealed that interferon-γ can reprogram white cell progenitors *in vivo* to express increased levels of the neutrophil cytochrome. Assessment of the efficacy of interferon-γ in management of CGD is being performed as part of an international controlled clinical trial.

III. Gene Transfer and Modification in Hematopoietic Cells.

In collaboration with Dr. David Williams (HHMI, Children's Hospital, Boston), Dr. Orkin has continued to develop strategies for the efficient transfer

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of genes to hematopoietic stem cells. Transfer of human adenosine deaminase (ADA) expression to mouse stem cells has been accomplished using recombinant retroviral vectors. Experiments have been initiated to disrupt the X-encoded cytochrome locus in cultured white cells by homologous recombination, in an attempt to explore the potential of gene transfer in correction of X-CGD. In addition, to evaluate the role of the erythroid nuclear factor GF-1, Dr. Orkin and his colleagues are examining the feasibility of targeting disruptions or modifications of the locus by recombination in cultured erythroid cells. Genetically altered, cultured hematopoietic cells may provide valuable reagents with which to explore the expression and biosynthesis of the superoxide-generating system in white blood cells and the control of gene expression in erythroid cells.

Dr. Orkin is also Leland Fikes Professor of Pediatric Medicine at Harvard Medical School and Children's Hospital, Boston.

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DEVELOPMENTAL DISORDERS IN TRANSGENIC MICE

PAUL A. OVERBEEK, Ph.D., *Assistant Investigator*

Research over the past year has made use of transgenic mice to study mammalian development. Two of the transgenic families under study (*downless* and *spermless*) have developmental abnormalities caused by insertional mutations. For both of these families, genomic sequences flanking the transgenic inserts have been cloned and used to search for the coding sequences of the inactivated genes. In addition to the insertional mutants, the α-crystallin promoter has been used to direct lens-specific expression in transgenic mice. Two different constructs have been studied in some detail: one encodes a truncated SV40 early region, the other codes for transforming growth factor α (TGF-α).

I. *downless*.

In one transgenic family, integration of the transgenic DNA inactivated the *downless* gene. This gene had been identified previously by classical genetics and had been mapped to mouse chromosome 10. Single-copy genomic sequences flanking the site of integration in the transgenic family were cloned and shown to map to mouse chromosome 10. The flanking sequences were used to isolate homologous clones from the wild-type genome. The wild-type clones did not overlap, implying that a deletion had accompanied integration of the transgenic DNA. To determine the size of the deletion, a restriction fragment analysis was done, using pulsed-field gel electrophoresis. The results indicate that there was a deletion of ~75 kb and that the deleted region contains a G-C–rich region, which could potentially represent the 5′ end of the *downless* gene. Bidirectional chromosomal walking from both flanks is in progress, and ~60 kb of genomic sequences has been isolated. The genomic sequences are being screened for evolutionary conservation, and the conserved sequences are being used as probes to screen a newborn skin cDNA library.

II. *spermless*.

In another transgenic family, integration of the transgenic DNA has inactivated a gene essential for normal sperm development. The mutation is recessive and leads to sterility in homozygous transgenic males. The fertility of the homozygous females is not affected. The mutation appears to block spermatogenesis at a specific stage of spermatid development. Mitotic and meiotic divisions appear to be unaffected, but development ceases at the round spermatid stage, prior to the onset of sperm tail synthesis. The localized and stage-specific nature of the defect suggests that a gene essential for a unique step in sperm maturation has been inactivated.

The genomic sequences flanking one side of the transgenic insert have now been cloned. These sequences were used for a restriction fragment length polymorphism (RFLP) analysis, which confirmed that the sterile males were homozygous for the transgenic insert. The sequences have also been mapped adjacent to the retinoblastoma locus on mouse chromosome 14. Current experiments are directed toward the cloning of the genomic sequences flanking the other end of the transgenic insert and the cloning of the corresponding regions of the wild-type genome.

III. Truncated SV40 Early Region.

Transgenic mice that express the full-length SV40 T antigen in the lens develop lens tumors. An interesting transgenic family was found to have integrated a truncated version of the T antigen. The mice show lens ablation rather than lens tumor formation. The truncation appears to have converted the T antigen from an inducer of cell proliferation to an inhibitor of cell proliferation. The transgenic insert, along with the adjacent mouse genomic sequences, has been cloned and sequenced. The T antigen–coding region was found to be truncated near amino acid 200 and fused to an open reading frame of 21 amino acids in the genomic sequences. In collaboration with Dr. Janet Butel, immunoprecipitation experiments revealed the presence of a protein of the appropriate size in lens extracts from the transgenic mice. The truncated T antigen retains the region that interacts with the retinoblastoma (RB) protein, while the region that binds to p53 has been lost. This observation suggests possible models for the mechanism of action of the altered T antigen. For instance, the modified T antigen may stabilize the antiproliferative function of the RB protein (perhaps by blocking phosphorylation). As a consequence, mitosis might be inhibited, resulting in lens ablation. Experiments have recently been initiated using tissue culture cells to test this model.

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The crystallin promoter linked to the truncated T antigen has been used to generate additional transgenic mice. The new families also have cataracts and microphthalmia, verifying that the lens ablation is a reproducible consequence of expression of the altered early region of SV40. The transgenic mice can be rapidly identified by simple visual inspection. Since the mice are not adversely affected by the cataracts, the construct has been modified to provide a dominant vector that can be used to simplify the identification of future transgenic mice.

IV. Transforming Growth Factor α.

Transgenic mice that express either TGF-α or epidermal growth factor (EGF) in the lens have recently been generated. The mice have readily apparent alterations in the architecture of their eyes. Anatomically the corneas are cloudy, the lenses are displaced anteriorly, and the anterior chambers of the eyes appear to be missing. Histological studies indicate that the growth factors induce aniridia and abnormal differentiation of the epithelial cells of the cornea. The TGF-α and EGF transgenic mice show identical phenotypes. These transgenic mice provide a unique developmental system in which it should be possible to study the mechanism by which expression of a growth factor in one cell type (in this case, the lens) can induce an altered pattern of morphogenesis in adjacent cells (the iris and cornea) during embryonic development.

Transgenic mice have also been generated to test promoters from the mouse rhodopsin, interstitial retinol-binding protein (IRBP), and tyrosinase genes. One goal is to identify regulatory sequences that can be used to direct transgene expression to a variety of different cell types in the embryonic eye. Future experiments can use these promoters to study communication between different cell types during development. The experiments will be designed to study the control of morphogenesis and cellular differentiation during embryonic development.

Dr. Overbeek is also Assistant Professor of Cell Biology and of Molecular Genetics at the Baylor College of Medicine.

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TRANSGENIC APPROACHES FOR STUDYING MOUSE DEVELOPMENT

RICHARD D. PALMITER, PH.D., *Investigator*

During the last year this laboratory has continued the productive collaboration with Dr. Ralph Brinster's laboratory at the University of Pennsylvania. These projects involve introduction of foreign genes into the germline of mice as a means of studying genetic phenomena in the intact animal. In addition to continuing studies aimed at 1) understanding the regulation of animal growth, 2) identifying the DNA elements involved in cell-specific gene regulation, and 3) analyzing oncogene-induced neoplasia of exocrine pancreas and liver, there has been progress in three new areas.

I. Homologous Recombination.

Targeting foreign DNA to specific chromosomal sites by homologous recombination provides an invaluable tool for studying gene function and correcting genetic defects. Although homologous recombination is the rule in some lower eukaryotes, nonhomologous integration of foreign DNA predominates in mammalian cells. The frequency of homologous events is ~0.1–1% of all integration events. If the frequency of achieving homologous recombination were 1% or better, then microinjection of DNA into mouse eggs would represent a direct way of introducing changes in specific genetic loci. Thus experiments were designed to attempt to correct a genetic defect in the class II major histocompatibility E_α locus that occurs in some strains of mice.

DNA molecules containing the 5′ end of a functional E_α gene were injected into mouse eggs bearing mutant E_α genes with a 630 bp deletion that encompasses the promoter and first exon. The deletion was corrected in 1 of ~500 transgenic mice that incorporated the injected DNA, and this corrected E_α gene was transmitted to progeny that were bred to homozygosity. Southern blot analysis, polymerase chain reaction amplification of the DNA spanning the deletion, and sequence analysis revealed that the corrected allele resembles the wild-type E_α gene. At sites of single–base pair polymorphisms, there was apparently random conversion of either the donor or recipient DNA sequence; in addition, many point mutations were introduced, suggesting that the recombination process was error prone. Messenger RNAs were produced from the corrected allele in a tissue-specific manner, but their sizes were different from the wild-type allele and they did not produce detectable E_α protein. This experiment demonstrates the feasibility of targeting foreign DNA to a gene that is completely inactive in fertilized eggs. However, because there was only one correction event after injecting >10,000 mouse eggs, the frequency cannot be calculated. Several aspects of this experiment that became evident during its execution suggested that the E_α gene may not have been an optimal choice for these experiments; hence current experiments are aimed at targeting DNA to another gene.

II. Regulation of Globin Gene Expression.

For many years, expression of globin genes in transgenic mice was either not achieved or achieved at very low levels. This was true for several reasons: 1) expression of the human β-globin gene was seriously inhibited by plasmid vector sequences that were included with most of the early constructs that were tested; 2) the enhancers for the human β-globin gene are located within and downstream of the gene, features that made analysis difficult; and 3) optimal expression of the β-globin gene depends on sequences located ~80 kb upstream of the gene. These sequences, now often referred to as the locus-activating region (LAR), were discovered because there are five sites within this region that are DNase I hypersensitive at all developmental stages in erythroid cells and because in certain thalassemias these sequences are deleted, leaving the β-globin gene and its enhancers intact. Frank Grosveld and his collaborators showed that when transgenic mice were made that contain the LAR along with the human β-globin gene, expression of each integrated gene copy was equivalent to an endogenous mouse β-globin gene. Subsequent experiments, in collaboration with Dr. Tim Townes (University of Alabama), revealed that pieces of DNA carrying individual hypersensitive sites could produce nearly the same result as the intact LAR. These sequences could also be used to achieve high-level expression of the human α-globin gene, a gene that had not been expressed in transgenic mice without the LAR. Co-injecting both the α- and β-globin genes with LAR sequences produced transgenic mice that express as much human hemoglobin as mouse hemoglobin, and the human globin is functional, as judged by oxygen dissociation measurements on human globin purified from these mice. These experiments demonstrate the feasibility of expressing mutant human hemoglobin mole-

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cules in transgenic mice as a means of producing models of human hemoglobinopathies.

III. Megacolon in Mice Expressing *Hox-1.4*.

Hox-1.4, a member of the cluster of homeobox-containing genes on mouse chromosome 6, is expressed almost exclusively in the testis of adult mice but is expressed in a number of organ systems during midgestation. This gene, like other homeobox-containing genes, is probably a transcription factor that regulates expression of other genes and possibly itself. A marked version of the *Hox-1.4* gene that would encode a functional Hox-1.4 protein but have an RNA transcript that could be identified was made in collaboration with Dr. Debra Wolgemuth (Columbia University). Several lines of transgenic mice expressed the marked *Hox-1.4* gene in appropriate sites during embryogenesis (as revealed by *in situ* hybridization) and in the germ cells of the adult testis. These experiments indicate that the sequences necessary for cell-specific expression lie within the 7 kb of *Hox-1.4* sequence that was tested.

The most remarkable effect of *Hox-1.4* expression in these transgenic mice is that the mice develop a condition known as megacolon. These mice have difficulty extruding feces; consequently the bowel becomes grossly distended, a condition that can cause death. This syndrome resembles that of two existing mouse strains and a congenital megacolon condition in humans known as Hirschsprung's disease. Megacolon is thought to be due to inadequate innervation of the distal portion of the colon by the enteric nervous system. The fact that *Hox-1.4* is expressed abundantly in the mesenchymal cells in the day 12.5 embryo suggests that the defect in these transgenic mice may lie in inappropriate pathfinding signals elaborated by mesodermal cells in the gut rather than a defect in the neural crest cells that migrate into the gut to form the enteric nervous system. These experiments provide an unexpected molecular clue to the development of the enteric nervous system that has stimulated research in this area.

Dr. Palmiter is also Professor of Biochemistry at the University of Washington.

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INSECT PATTERN FORMATION

NORBERT PERRIMON, PH.D., *Assistant Investigator*

Dr. Perrimon's laboratory is studying the molecular mechanisms of cellular differentiation during pattern formation. The goal is to use genetic and molecular approaches to define networks of interacting genes that are involved in the elaboration of specific pattern elements. Two patterning systems, embryonic segmentation and neurogenesis, are being investigated, with the belief that similar gene networks may be operating in both systems.

I. Role of *l(1)pole hole* in Signal Transduction.

The function of a number of maternally encoded gene products is to direct the expression of zygotic genes. The molecular mechanisms underlying early determinative processes rely on the interactions between maternally stored and zygotically activated gene products. One goal has been to study the role of the *l(1)pole hole* [*l(1)ph*] gene in establishing the spatial coordinates of the embryo. This laboratory has shown that *l(1)ph* encodes a single 3.2 kb RNA that has homology to the mammalian *raf* proto-oncogene, which is a serine-threonine kinase. Embryos derived from females that lack the *l(1)ph* wild-type gene product are missing their most anterior and posterior structures. A similar embryonic phenotype is observed in embryos derived from females homozygous for the *torso* mutation. The *torso* gene has recently been cloned by Dr. Nusslein-Volhard's group and was found to share extensive amino acid homology with a membrane-bound tyrosine kinase. Genetic epistasis experiments suggest that *l(1)ph* acts downstream of *torso*. One current model suggests that the mammalian c-*raf* acts as a "molecular bottleneck," serving as a signal transducer for a variety of growth factor receptors with tyrosine kinase activity. In response to extracellular signals, c-*raf*, which is located at the membrane, is phosphorylated on a subset of its tyrosine residues and is thereby activated. After activation, c-*raf* moves to the nucleus, where it participates in the control of gene expression. It is therefore postulated that the *l(1)ph* gene product is phosphorylated by the *torso* gene, thereby mediating the *torso* information. This hypothesis is being tested using polyclonal antibodies against the *l(1)ph* gene product and phosphorylation assays. The combination of biochemical and genetic approaches should allow the understanding of the role of c-*raf* [i.e., *l(1)ph*] in signal transduction.

II. Mechanisms of Intrasegmental Patterning.

Once a group of cells is assigned to a specific germ layer, both cell lineage and cell-cell interactions define the particular identities of single cells. The establishment of intrasegmental patterning provides an attractive system to study these mechanisms. This laboratory has identified three new segment polarity loci, *dishevelled* (*dsh*), *porcupine* (*porc*), and *zeste-white-3*, that are associated with maternal-effect lethal phenotypes that affect the fates of rows of cells within every segment. Another segment polarity gene, *wingless* (*wg*), has been studied and found by others to be the *Drosophila* homologue of the murine oncogene *int-1*, whose misexpression can result in mammary tumors. The *wg* gene has been shown to act nonautonomously and is believed to encode a short-range morphogenetic signal. The receptor(s) for such a signal is likely to be required in most cells, and when the gene encoding the receptor is mutated the embryonic phenotype should be identical to that of *wg*. Identified by genetic means, *porc* and *dsh* are two likely candidates for "receptor" gene. A battery of clonal analysis experiments has allowed the demonstration of the cellular autonomy of *dsh*. An investigation has revealed that *porc*, like *wg* and unlike *dsh*, is not cell autonomous. The present model is that *porc* is required for production of the *wg* signal and that *dsh* is required to receive or transduce it. Immunological approaches are being used to examine (in collaboration with Dr. R. Nusse) the pattern of *wg* protein expression in both *porc* and *dsh* mutants. Molecular characterization of both genes is in progress and will likely culminate with the characterization of gene products involved in the *D-int1* transducing machinery.

III. Ventral Midline as a Developmental System.

Dr. Perrimon's laboratory is studying the determination of a set of cells that are located along the ventral midline of the embryo. Affecting these midline cells are seven loci that appear to play important roles. Genetic and molecular analyses of three of these loci, *orthodenticle* (*otd*), *midline*, and *spitz*, are in progress. Within the central nervous system (CNS) these genes affect the development of both neural (midline neurons) and nonneural (midline glia) cells that play key roles in the forma-

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tion of the embryonic axonal tracks. The *otd* mutations that completely eliminate gene activity result in the fusion of pairs of horizontal axonal commissures present in each segment of the embryo. Most likely this defect is secondary to the abnormal differentiation of a small subset of neurons that are normally located at the ventral midline in wild-type embryos. Less-severe mutations in this gene do not affect the embryonic CNS but act later in development, causing a deletion of specific visual structures, the ocelli, in the adult fly. In collaboration with Dr. Allan C. Spradling (HHMI, The Carnegie Institution of Washington), this laboratory has cloned the *otd* gene. The *otd* gene encodes one major 5 kb message that is expressed in precursor cells along the ventral midline. Sequence analysis indicates that *otd* encodes a protein with a highly repeated structure that contains a homeodomain that has been implicated in DNA-binding activities. The analysis of *otd* is providing insights about a gene product that is essential not only for embryonic axonal patterning but also for the determination of eye structures during imaginal development.

IV. Targeting Gene Expression in *Drosophila*.

Although most of the work in Dr. Perrimon's laboratory has been focused on the analysis of developmental genes, a system is also being developed that will allow the controlled expression of specific genes in predetermined cells or groups of cells. The approach taken utilizes the activation properties of the yeast GAL4 protein, which activates only those genes bearing a GAL4-binding site within their promoters. It has been shown by others that GAL4 can also activate transcription in *Drosophila*. This approach involves the expression of GAL4 in a subset of cells and the subsequent introduction of genes whose transcription is driven by GAL4-binding sites. Some of the applications of this system will involve the analysis of pattern regulation after selective killing of specific cells and the analysis of ectopic expression of "switch" genes, which control cell fate or identity.

Dr. Perrimon is also Assistant Professor of Genetics at the Harvard Medical School.

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PKD1 AND ESSENTIAL HYPERTENSION: A MOLECULAR GENETIC APPROACH

STEPHEN T. REEDERS, M.D., *Assistant Investigator*

The molecular genetics of inherited renal disease and hypertension are the focus of Dr. Reeders' laboratory. A major element of this work is the development of methods to clone genes (from a knowledge of the segregation patterns of mutant phenotypes) when the biochemical and cellular functions are unknown.

I. Autosomal Dominant Polycystic Kidney Disease (ADPKD).

ADPKD is one of the most common autosomal dominant diseases of humans, affecting 1 in 1,000. Progressive cystic dilation arises from all levels of the nephron and progresses throughout life. Gradual impairment of renal function leads to the need for dialysis and transplantation, usually in the fifth or sixth decades. The underlying biochemical abnormality is not known, although there is increasing evidence that the primary pathology is tubular epithelial hyperplasia.

Previous work in this laboratory has demonstrated that mutations in at least two genes cause ADPKD. Most of these mutations disrupt the PKD1 gene, which was previously mapped by Dr. Reeders to the p13 band of chromosome 16. A series of chromosome 16–only interspecies hybrids has been created to facilitate the rapid mapping of new DNA clones to the 16p13 region. As a result, several new clones have been mapped within an interval of 2–4 megabases (Mb) surrounding the PKD1 gene. Genetic analysis has identified flanking markers that are <3 centimorgans apart. Pulsed-field gel analysis has led to the construction of a 2 Mb long-range restriction map of the region between and beyond the flanking markers and has allowed the PKD1 gene to be localized to a region of ~650 kb. To expedite the recovery of coding sequences from this region, an attempt has been made to map and clone CG-rich islands that are frequently associated with the 5′ ends of coding sequences, especially those of housekeeping genes. Ten CG-rich islands have been mapped within the 650 kb, and five of these have been cloned. Three probes encompassing CG-rich islands have been used to obtain cDNAs from adult kidney libraries. So far, no mutation of these coding regions has been identified in patients.

II. Essential Hypertension.

Essential hypertension affects 50 million Americans and contributes to 1 million deaths annually. A major element of the susceptibility to hypertension is inherited. Risk of hypertension is not, however, inherited as a simple Mendelian characteristic but as a complex polygenic trait. Thus hypertension provides a critical test of the power of genetic linkage and molecular genetic methods for detection of multiple "risk alleles."

Dr. Reeders is employing several techniques in an attempt to unravel this complex trait. A major approach is to study the genetic control of red blood cell sodium-lithium countertransport (RBCT), a function that is probably performed by one or more sodium-hydrogen antiporters. RBCT correlates broadly with blood pressure and is probably the best available marker for essential hypertension. In collaboration with Dr. Chris Dudley (University of Oxford), Dr. Reeders has studied the segregation of polymorphic loci from chromosome 1p35-36 flanking an Na-H antiporter (APNH) in six families in whom multiple individuals were found to be hypertensive. RBCT levels were measured. The APNH locus did not appear to segregate with hypertension or RBCT, indicating that this locus does not contribute to either blood pressure variability or RBCT levels in a major way in these families.

A genomic cosmid clone containing most of the APNH gene was obtained to permit the detection of increased polymorphism at this locus and to allow study of the organization of the APNH gene.

Dr. Reeders is also Assistant Professor of Internal Medicine and of Human Genetics at Yale University School of Medicine.

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MOLECULAR ANALYSIS OF DEVELOPMENT IN *DROSOPHILA MELANOGASTER*

SHIGERU SAKONJU, PH.D., *Assistant Investigator*

Dr. Sakonju's laboratory is interested in understanding how homeotic gene products determine the fate of individual cells in body segments of *Drosophila melanogaster*. Much of the work has focused on *abd-A* and *Abd-B*, two homeotic genes in the bithorax complex that specify the identity of abdominal segments. Analysis of the *abd-A* and *Abd-B* gene structures has provided insights into the molecular functions of these genes. This knowledge is being used to investigate the molecular mechanisms by which the homeotic proteins regulate the expression of their target genes.

I. Molecular Analysis of the *abd-A* Gene.

The *abd-A* gene is required for the identity of parasegment (PS) 7 through PS13 (corresponding to the posterior part of the first through the anterior part of the eighth abdominal segments). The complete gene structure of *abd-A* has been determined. The gene encodes a 4.8 kb transcript derived from six exons spread over 23 kb of genomic DNA. Transcription of *abd-A* initiates from a cluster of three sites separated by a few bases. The *abd-A* transcripts contain a single long open reading frame (ORF). Translation of the ORF yields a 36 kDa protein containing a highly conserved homeodomain and a cluster of glutamine residues, the so-called *opa* or M repeats. Unlike *Ubx* and *Abd-B* (the other two genes within the bithorax complex), multiple transcripts are not produced by alternative splicing of the *abd-A* transcripts. Thus the diverse genetic functions attributed to *abd-A* must be carried out by the sole product of this gene.

II. Regulation of Transcription by the *abd-A* Gene Product.

Homeotic gene products are assumed to function by binding to DNA sequences and regulating the expression of target genes. This assumption is derived from *in vitro* DNA-binding studies and from experiments using tissue culture cells. One goal of Dr. Sakonju's laboratory is to test if this assumption is correct *in vivo* during *Drosophila* development. Genetic analysis indicates that *Antennapedia* (*Antp*) gene expression is negatively regulated by *abd-A* in the abdominal segments of *Drosophila*. Furthermore, Dr. Anne Boulet and Dr. Matthew P. Scott (HHMI, University of Colorado, Boulder) have shown that the repression by *abd-A* is mediated through 1.4 kb of DNA upstream from the *Antp* P2 promoter. Hence this promoter was chosen as a potential *in vitro* and *in vivo* target site for the *abd-A* gene product. The *abd-A* protein was overproduced and purified from bacteria. This protein was first shown to immunoprecipitate a restriction fragment located ~900 bp upstream of the *Antp* P2 start site. The precise location of DNA binding was then determined, using a footprint analysis. The binding site includes two stretches of the consensus sequence derived from other homeodomain-associated proteins. To demonstrate that this binding site is essential for *in vivo* repression by *abd-A*, it is being deleted from the *Antp* P2 upstream DNA. The DNA will then be fused to β-galactosidase, introduced using the P element, and tested to see if this construct escapes the repression in the abdominal segments of the wild-type *Drosophila*.

III. Effects of *iab-4* Mutations on the Gonad Development.

Lesions in the *iab-4* region of the *abd-A* domain are thought to disrupt cis-regulatory regions required for proper regulation of the *abd-A* transcript. The *iab-4* mutants show a transformation of the fourth abdominal segment toward the third. In addition, they are sterile due to a loss of gonads. To define the specific step during the gonad formation that is affected by the *abd-A* mutation, Dr. Sakonju's laboratory (in collaboration with Dr. Janos Szabad) has characterized the gonadal defect in more detail. Pole cell transplantation experiments and antibody staining of pole cells in both wild-type and mutant embryos indicate that 1) the defect is somatic, not germline, dependent, and 2) in *iab-4* mutant embryos the pole cells migrate normally, but the gonadal sheath that surrounds the pole cells is never formed. Genetic mosaic analysis is being carried out to determine which type of cells (mesodermal, ectodermal, or both) must be mutant to produce the mutant gonadal phenotype.

IV. Molecular Analysis of the *Abd-B* Gene.

Genetic analysis has revealed two separable functions within the *Abd-B* gene: 1) the morphogenetic

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function determines the identity of PS10 to PS13, and 2) the regulatory function suppresses the activities of other homeotic genes in PS14. Transcriptional analysis of *Abd-B* has revealed four overlapping transcripts that are generated from the *Abd-B* region. All four transcripts contain a unique 5′ exon but share several 3′ exons. Promoters for two of the transcripts (4.6 and 3.4 kb) have been identified. The 4.6 kb RNA initiates ~25 kb downstream of the 3.4 kb RNA initiation site. Two other transcripts initiate farther upstream of these promoters. Although the *Abd-B* transcripts initiate from multiple promoters, they are polyadenylated at the same two sites. DNA sequence analysis of the *Abd-B* cDNAs indicates that the 4.6 kb RNA encodes a 55 kDa protein; a 30 kDa protein is produced by three other transcripts. Both proteins contain a homeodomain, but they differ by the presence or absence of a 25 kDa domain on the amino terminus. *In situ* hybridization and immunofluorescence techniques are being used to localize RNA and protein products in both the wild-type and mutant *Drosophila* embryos. The results support the assignment of the morphogenetic function (active in PS10–13) to the 55 kDa protein and the regulatory function (active in PS14) to the 30 kDa protein.

Dr. Sakonju is also Assistant Professor of Human Genetics at the University of Utah School of Medicine.

GENETIC CONTROL OF PATTERN FORMATION

MATTHEW P. SCOTT, PH.D., *Associate Investigator*

The pattern of structures formed by a developing embryo is controlled by a set of regulatory genes that specify how cells are to divide, move, interact, produce, and localize particular molecules. Many of the relevant regulatory genes have been identified in *Drosophila*, including the segmentation genes that govern subdivision of the embryo into the proper number of correctly patterned segments and the homeotic genes that direct the formation of the structures that make the different segments unique. It is now crucial to learn how the regulatory genes interact and how the products of the genes control cellular differentiation and pattern formation. There is precise temporal and spatial control of the transcription of many of the regulatory genes, and it is often found that the places where a gene is expressed correspond well with the parts of the embryo that are affected by the loss of the gene's functions. Dr. Scott's laboratory is addressing two key questions: How is the spatial and temporal regulation of segmentation and homeotic gene expression attained? How do the spatially restricted proteins carry out their functions?

I. Gene Structure and Control Elements of Segmentation and Homeotic Genes.

Segmentation genes are expressed in striking patterns of transverse stripes in blastoderm stage embryos and in more complex patterns in the developing nervous system. At the blastoderm stage, homeotic genes are expressed in simple patterns, such as in all the cells of one or two segment primordia, but the patterns of homeotic gene transcription become extremely complex later in development. Sandra Sonoda has used regulatory sequences from the *fushi tarazu* (*ftz*) segmentation gene in experiments in which a *lacZ* gene is fused to the control sequences and introduced into *Drosophila*. She has demonstrated that the binding site in the *ftz* gene for a protein that has been purified by a collaborator, Dr. Carl Wu (National Institutes of Health), is important for activation of the expression of *ftz* in stripes. The properties of the binding site are being further explored.

John Bermingham and Matthew Petitt have analyzed the expression of the two *Antennapedia* (*Antp*) homeotic gene promoters. They have used *in situ* hybridization of promoter-specific probes to whole-mount embryos and sectioned embryos to reveal the different patterns of expression of the two promoters and to examine the changes in transcription from both promoters in embryos that are mutant for certain regulators of *Antp* or for *Antp* itself. Thus the two promoters are regulated differently. Dr. Anne Boulet has found that 10 kb upstream of the second *Antp* promoter is sufficient for a partially normal pattern of expression of a *lacZ* construct and has begun to identify cis-acting elements responsible for different aspects of the expression pattern. Dr. Deborah Andrew has studied the structure of the homeotic gene, *Sex combs reduced* (*Scr*), that is adjacent to *Antp* on the chromosome and has done an evolutionary comparison of some of the promoter sequences. She has found that *Scr* also has two promoters, but both promoters are expressed in the same pattern. Possible regulatory elements in the *Antp* gene have been identified by Dr. Joan Hooper, who has compared the 103 kb gene from *Drosophila melanogaster* with the even larger gene from *D. virilis*. Highly conserved sequences are present in many regions within and upstream of the transcription units; these conserved regions will serve to guide the construction and deletion analyses of the *lacZ* fusions. John Bermingham has analyzed the alternative RNA splicing patterns of *Antp* and has found that four somewhat different proteins are encoded by the gene's transcripts.

II. Homeodomain Proteins Are Transcription Factors.

Mutations in homeotic genes cause dramatic transformations, such as the development of legs where the antennae should be or of antennae where the mouthparts should be. These erroneous choices of developmental pathway can be due to a single protein expressed in the wrong place or to the absence of a single protein from the cells where it normally functions. A single protein must therefore be capable of affecting many cellular processes. Dr. Rolf Reuter has been examining the functions of homeotic proteins in internal tissues and has discovered cases where a relatively simple morphological event results from the presence of a homeotic protein in certain cells. The goal is to understand the cell biology of the transformations and obtain clues as to what sorts of genes might be coordinately regulated by homeotic proteins. Another ap-

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proach to the same question has been to study the molecular functions of the homeotic proteins, which has led to the recognition that they are transcription factors.

Many of the regulatory genes that control division of the *Drosophila* embryo into segments and many of the homeotic genes that control what structures will develop in different regions of the embryo have been found to contain homeoboxes, 183 bp DNA sequences that encode evolutionarily conserved 61–amino acid protein domains. Similar homeodomain proteins are found in *Drosophila* and in most other animal species that have been examined for the sequences, including humans. In all cases the homeodomains are similar in structure to bacterial DNA-binding proteins, and in several cases they have been shown to bind to DNA in a sequence-specific manner. These results suggested that homeodomain-containing proteins might regulate a largely unknown set of target genes by binding to their DNA and affecting their transcription. Gary Winslow and Dr. Shigeo Hayashi have used a cultured cell system to test this hypothesis and have shown that three of the *Drosophila* homeodomain proteins, the products of the *Antp* and *Scr* homeotic genes and the *ftz* segmentation gene, are capable of activating transcription of a variety of synthetic target genes. The target genes used were constructed using sequences that Dr. Hayashi had shown are bound *in vitro* by the protein encoded by the *Antp* homeotic gene. Therefore these homeodomain proteins are capable of controlling transcription, and the task now is to understand what determines their specificity of action. The *Scr* gene is required for the proper development of the posterior head and anterior thorax, whereas the *Antp* gene is required for all of the thoracic segments to develop thoracic structures rather than more anterior structures. The *ftz* gene is required much earlier and is necessary to direct the division of the embryo into the correct number of segments. The homeodomains of the proteins are very similar, suggesting that the specificity of action may be due to the other parts of the proteins, which are quite distinct. Modified forms of the proteins are being constructed and expressed in cultured cells and in *Drosophila* to see the effects of the modifications on their functions. The exact structure of the target gene can also affect the activity of the homeodomain proteins, and variations on target gene structure are being tested in cultured cells to determine what aspects of the cis-acting sequences are important for function.

III. Newly Characterized Homeotic Genes.

Dr. John Tamkun (now at the University of California at Santa Cruz) has been collaborating with Dr. James Kennison (National Institutes of Health) in a study of new homeotic genes that were discovered because their alleles act as enhancers or suppressors of known homeotic mutations. The new genes that have been most studied, *brahma* (*brm*) and *kismet* (*kis*), have different properties. The *brm* gene is required both during oogenesis and in the embryo. When *brm* function is drastically reduced, there are severe defects in embryogenesis, suggesting that *brm* is required for the proper function of other regulatory genes in the early embryo. In contrast, *kis*, alleles of which can cause homeotic transformations in adult *Drosophila*, may be a gene that is regulated by homeotic genes such as *Antp* or may encode a cofactor necessary for the function of other homeotic proteins. The *brm* gene has been cloned and sequenced; the protein sequence has no relation to other reported proteins. The *brm* transcripts are expressed ubiquitously.

IV. A Transmembrane Protein Encoded by a Segmentation Gene.

Not all of the segmentation genes encode transcription factors. Dr. Hooper has been investigating the gene *patched* (*ptc*), which acts in every segment of the developing embryo to control intrasegmental pattern formation. In the absence of *ptc* function the central part of each segment's pattern develops structures that are a mirror image of the anterior part of the segment. Cells in the center of the segment appear to be confused about where they are and which direction is anterior. The gene was cloned and found to encode a protein that is predicted to have between 7 and 12 transmembrane domains. Therefore it is likely that the *ptc* protein is involved in cell-cell communication processes that inform cells of their positions within the segment primordium and of the orientation of the cell in relation to the embryo as a whole. The expression pattern of *ptc* is elaborate. Transcripts are first nearly everywhere, then disappear from the posterior of each segment primordium, and then disappear from the center of the anterior of each segment. The final pattern of 30 transverse stripes is one of the most complex known for a segmentation gene. In the nervous system, *ptc* is also required for proper patterning of cells. Together with the segmentation gene products that

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control transcription of other genes, cell communication components such as the *ptc* protein guide the development of pattern in multiple embryonic tissues.

Dr. Scott is also Associate Professor of Molecular, Cellular, and Developmental Biology at the University of Colorado at Boulder and Associate Professor at the University of Colorado Health Sciences Center.

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MOLECULAR BASIS OF FAMILIAL HYPERTROPHIC CARDIOMYOPATHY AND INSULIN-DEPENDENT DIABETES

JONATHAN G. SEIDMAN, PH.D., *Investigator*

I. The Locus Encoding the Familial Hypertrophic Cardiomyopathy Gene Maps to Chromosome 14q1.

Familial hypertrophic cardiomyopathy (HC) is an idiopathic heart muscle disorder with an autosomal dominant pattern of inheritance. The disease is characterized clinically by myocardial hypertrophy, a wide spectrum of symptomatic involvement, and a 2–4% annual mortality rate from sudden death, which can even occur in the asymptomatic individual. Postmortem examination reveals increased myocardial mass with myocyte and myofibrillar disarray. Diagnosis is based on typical clinical features and the two-dimensional echocardiographic demonstration of unexplained left and/or right ventricular hypertrophy. Diagnosis is often complicated in the young because hypertrophy may not develop until after adolescent growth has been completed. The anatomical distribution of myocardial hypertrophy and severity of symptoms may be variable, even within a family. During the past 30 years the cardiac features of this disease have been extensively reported, but the etiology and molecular pathophysiology have remained speculative.

To understand the genetic basis for familial HC, Dr. Seidman used a molecular genetic technique to identify the chromosomal position of the disease locus in a large family. This approach has been used by others to identify the genetic loci responsible for more than 30 inherited disorders, including Huntington's disease, familial polyposis, cutaneous malignant melanoma-dysplastic nevus syndrome, ataxia-telangiectasia, and Duchenne muscular dystrophy. Cosegregation analysis, which defines genetic linkage between a known chromosomal location (defined by a DNA probe) and the disease gene, has been used to map each disease locus to a region of the human genome. Because there were neither candidate genes nor cytogenetic abnormalities to suggest the chromosomal location of the gene responsible for familial HC, Dr. Seidman and his colleagues began to screen DNA probes corresponding to loci throughout the genome, to identify one that is linked to the familial HC locus. These DNA probes recognize restriction fragment length polymorphisms (RFLPs) and are a subset of DNA markers used to construct a genetic linkage map reported to span the human genome.

A large family in which the familial HC gene segregated as an autosomal dominant trait was clinically evaluated and used in genetic analyses. Forty-one polymorphic DNA probes were used before identifying one (CRI-L436, derived from chromosome 14) that is closely linked to the familial HC locus in this family. No instances of recombination between the familial HC locus and the D14S26 locus (defined by CRI-L436) were observed. Statistical analysis of the disease status and CRI-L436 alleles in 41 unaffected members and 20 affected members of this family suggests that the odds are >2,000,000,000:1 (lod score = +9.37 at J = 0) that the familial HC gene locus is genetically linked to D14S26.

Chromosomal location of the disease locus suggests candidate genes that may be responsible for familial HC and provides a basis for determining whether additional genetic loci can independently cause this disorder in unrelated families.

II. Autoimmunity in Non-Obese Diabetic Mice Bearing a Functional T Cell Receptor β-Chain Transgene.

The NOD (non-obese diabetic) mouse develops spontaneous insulin-dependent diabetes mellitus characterized by infiltration of mononuclear cells, around and into the pancreatic islets, followed by beta cell destruction, similar to human type I diabetes. Diabetes susceptibility is associated with at least three recessive genes, one of which maps to the class II region of the major histocompatibility complex (MHC). There is a large body of evidence indicating that type I diabetes in animal models is T cell mediated. T cells predominate in the insulitis lesion, and diabetes can be prevented in NOD mice with therapies directed against T cells, such as neonatal thymectomy and the introduction of the *nu/nu* gene, which produces profound T cell immunodeficiency. Furthermore, overt disease can be adoptively transferred into healthy NOD neonates and preirradiated adult male recipients using purified T cells from diabetic mice. Disease can be transferred by islet-specific T cell lines. Evidence for T cell involvement in disease pathogenesis along with the selective nature of beta cell destruction has led to speculation that specific T cell receptor (TCR) sequences may be important for lymphocyte

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targeting to the lesion and the initiation of the autoimmune process.

The polypeptide α- and β-chains of the TCR provide critical contact points with MHC and antigen and form the basis of T cell recognition. Assembly of the functional α- and β-chain genes occurs during T cell development in the thymus by a process of recombination of germline DNA segments. Once a functional receptor is produced, further T cell gene rearrangements cease, a phenomenon referred to as allelic exclusion. Support for this model has been provided by studies conducted by Dr. Seidman and others, who have shown that transgenic mice bearing a functionally rearranged β-chain gene into the germline of mice prevent expression of the endogenous β-chain gene repertoire.

To examine the role of the endogenous T cell receptor repertoire on the development of insulitis and the production of autoantibodies in the NOD mouse, Dr. Seidman and his co-workers bred a functional β-chain transgene into the NOD mouse. During earlier studies a functional β-chain gene from the DO.11 hybridoma was introduced into B6xSJL mice. These transgenic mice were mated to NOD animals and four backcross generations were produced (transgene-bearing mice were selected for further mating in each generation). As expected, the transgene is fully functional in the backcross mice. Nearly all of the T cells in these animals bear the Vβ8 determinants encoded by the transgene. Furthermore, the transgene produces allelic exclusion of other Vβs from the surface of T cells in both the thymus and spleen.

Surprisingly, the transgene has no detectable effect on the development of autoimmunity in the NOD mouse. Autoantibodies against insulin precede the development of overt type I diabetes in humans and NOD mice, and the magnitude of the titers correlates with the progression to diabetes. The transgene did not significantly affect either incidence or titer of insulin autoantibodies; 4/15 (27%) of the transgene-positive mice compared with 4/21 (19%) of the transgene-negative mice expressed insulin autoantibodies similar to what is seen with NOD mice. Furthermore, the frequency of insulitis among transgene-bearing mice and non-transgene-bearing littermates was not significantly different (3/12 vs. 4/24), despite the fact that the transgene-bearing T cells were found in large numbers in the insulitis lesions of transgenic mice. These studies question the role of TCR specificity in the development of autoimmune diabetes.

Dr. Seidman is also Professor of Genetics at Harvard Medical School.

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MOLECULAR GENETICS OF THE HUMAN SEX CHROMOSOMES

LARRY J. SHAPIRO, M.D., *Investigator*

Research in Dr. Shapiro's laboratory is directed primarily toward elucidating the biology of the human sex chromosomes. The organization of genes on the X and Y chromosomes and the evolutionary history of these two structures is under study. The role of the sex chromosomes in primary sex determination and the functional regulation of gene expression of X-encoded genes through X inactivation are also major areas of interest. Investigation is proceeding into the basis of X-Y recombination in the pseudoautosomal region of the sex chromosomes and the generation of populational polymorphism in the sex chromosomes. In addition, one gene that encodes the enzyme steroid sulfatase (STS) is receiving particular scrutiny. The biosynthesis, post-translational processing, and targeting of this protein to subcellular organelles is the subject of a number of experiments. The evolutionary history of this gene, the mechanism by which it escapes regulation by X chromosome inactivation, and the significance of frequent deletions of this locus in human populations is receiving detailed consideration.

I. Organization of the X and Y Chromosomes.

It is commonly believed that the very dissimilar X and Y chromosomes evolved from a pair of homologous ancestral chromosomes. The finding that some modern day vertebrates lack sex chromosomes or a genetic mechanism of primary sex determination supports this view. One example is provided by certain fish that can change gonadal and phenotypic sex in response to environmental cues. Studies of the expression of putative regulatory genes in this dynamic system are under way. Pairing and segregation of the human X and Y chromosomes is facilitated by a chromosomal domain of sequence identity, the pseuduoautosomal region. In turn, this sequence homogeneity is maintained by frequent meiotic recombination between the X and Y chromosomes in this region. Absence of the pseudoautosomal region results in a complete failure of pairing of the X and Y during spermatogenesis. Studies of the pseudoautosomal, sex chromosome–unique boundaries in primates have provided evidence that gross chromosomal rearrangements, which include a pericentric inversion, have been responsible for creating this sharp junction. It is suspected that the pseudoautosomal region gradually expands through nonhomologous recombination and contracts through processes of chromosomal rearrangement over evolutionary time. Several consequences of this are the presence of sequence similarities between the X chromosome short arm and the Y chromosome long arm, which could be the basis for X/Y translocations encountered clinically. At least two genes (as well as a number of anonymous sequences) that map to the short arm of the X have pseudogenes on the Y chromosome that also permit observation of the processes by which genes, not under selective pressure, degenerate. More recent studies of a highly polymorphic family of sequences on the X chromosome and a related group of nonpolymorphic sequences on the Y chromosome should permit investigation of the role of genetic recombination in generating certain kinds of polymorphism.

II. Deletions of the Short Arm of the Human X Chromosome.

Deficiency of STS enzymatic activity produces a visible cutaneous abnormality called ichthyosis. This common inborn error of metabolism in man has an X-linked pattern of inheritance and affects as many as 1 in 2,000 males. Most often the clinical abnormalities are limited to ichthyosis, but occasionally more complex phenotypes are observed. At least 90% of STS subjects have substantial deletions of the X chromosome short arm that may involve contiguous genes to produce the more complicated clinical situations. Frequent deletions also seem to occur at another X short arm site, the dystrophin locus, resulting in Duchenne muscular dystrophy. Dr. Shapiro and his colleagues have constructed a detailed, rare cutting enzyme restriction map of the distal X short arm, using pulsed-field gel electrophoresis and cloning segments of this region as yeast artificial chromosomes. This information has been used to localize many of the breakpoints in these deletions. The deletions range from 40 kb to well over 1 Mb in length. Although the breakpoints are heterogeneous, they appear to cluster in a few regions. One deletion junction has been cloned and sequenced; no repetitive elements or secondary structures are present at the breakpoint, but an 8 bp direct repeat was introduced at the junction. Additional deletion breakpoints are being analyzed to gain further insight into the mechanisms that

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produce them. In particular, the possible role of an X chromosome–unique, dispersed, moderately repetitive element is being studied. Furthermore, this laboratory is attempting to determine whether these deletions occur more commonly in male or female germ cells and whether they arise preferentially during meiosis or during mitotic division of primordial germ cells. An effort to reproduce these deletions in the cultured somatic cell system is also under way.

III. Steroid Sulfatase Biosynthesis, Processing, and Regulation.

STS is a ubiquitously distributed enzyme. Recent data from the cloning of several other sulfatases show that STS is part of a multigene family. However, while most of the other sulfatases are localized in lysosomes, STS is not thought to reside in this cellular compartment. *In situ* hybridization, immunocytochemistry, and immunoelectron microscopy have been used to study the sites of synthesis and the localization of STS, which is predominantly found on the rough endoplasmic reticulum (RER). Manipulation of suitable expression constructs and studies of post-translational processing should clarify those structural features that direct STS to the RER and not to the lysosome. Furthermore, insight may be gained with regard to the pathogenesis of a new autosomal recessive human inherited condition, multiple sulfatase deficiency disease. In this condition, activities of many of the different sulfatase enzymes are coordinately reduced, probably through a mechanism that enhances the rate of turnover of these proteins. Finally, the signals that abruptly cause STS transcription to begin after differentiation of placental cytotrophoblast cells into syncytial trophoblast cells are being explored in a cell culture model.

IV. Regulation of X-encoded Gene Expression.

An additional area of interest in the laboratory is the regulation of gene expression via X chromosome inactivation. Several years ago, Dr. Shapiro's laboratory showed that X inactivation involves DNA methylation and that there are several genes that escape inactivation in a position-independent manner. The structure and function of promoters that are subject to X inactivation are being compared with those that are not so regulated to see if differences in expression can be ascribed to this region. In addition, the role of DNA-binding proteins in the X-inactivation process is being studied by looking for the induction of new sequence-specific factors after differentiation of teratocarcinoma cells *in vitro*. The effect of methylation on the binding of such proteins is also being evaluated.

Dr. Shapiro is also Professor of Pediatrics and Biological Chemistry at the University of California School of Medicine at Los Angeles.

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REGULATION OF GENE ACTIVITY IN B CELLS

HARINDER SINGH, PH.D., *Assistant Investigator*

Differential gene activity is a fundamental feature of cellular differentiation. Research in the laboratory of Dr. Singh focuses on transcriptional regulatory circuits that underlie the establishment and maintenance of cell-specific patterns of gene expression during mammalian development. The laboratory uses transformed B lymphocytes representing various stages of B cell differentiation as models to explore the molecular basis of differential gene activity in a defined lineage.

The heavy- and light-chain genes encoding the immunoglobulin (Ig) molecule are selectively transcribed in B cells. These genes are assembled from gene segments through an ordered series of somatic recombination events that occur in a developing B cell. Three B cell–specific, cis-acting transcriptional regulatory elements have been identified. Two of these elements are located in the introns between the variable and constant regions of the heavy- and κ-light-chain genes and act as enhancers. The third element is found upstream of both heavy- and κ-light-chain gene promoters.

The elaboration of a sensitive DNA-binding assay has led to the identification and characterization of two B cell–specific regulatory proteins. One of these proteins, Oct-2 (NF-A2), recognizes the octanucleotide sequence ATTTGCAT, which confers B cell specificity to Ig gene promoters. The same sequence motif is also a functional component of the heavy-chain gene enhancer. The second B cell–specific regulatory protein, NF-κB, recognizes the sequence GGGGACTTTCC, which appears to dictate the B cell specificity of the κ-gene enhancer. In contrast with the lineage-restricted Oct-2 protein, NF-κB exists in an inactive but inducible form in a variety of non–B cell types.

A novel expression screening strategy that uses recognition site probes was previously developed by Dr. Singh and his colleagues to enable the rapid isolation of genes encoding transcriptional regulators. This strategy was used to isolate cDNA clones for an NF-κB–like protein and the Oct-2 protein. The deduced amino acid sequences of these proteins reveal that the former belongs to the family of zinc finger proteins, whereas the latter contains a domain related to the homeobox.

Two different human Oct-2 cDNA clones have been isolated in the laboratories of Drs. David Baltimore and Phillip Sharp. The clones encode nearly identical proteins that differ at their carboxyl termini by 12 amino acids. In addition to the homeodomain, the predicted proteins contain a region, termed the POU-specific box, that defines a new class of regulatory proteins, as well as a putative leucine zipper domain. At least five different Oct-2 transcripts, ranging in size from 1.5 to 7 kb, are expressed specifically in various B cell lines. These transcripts appear to represent alternatively spliced mRNAs.

Because the regulatory functions of the various Oct-2 gene products, as well as the regulation of Oct-2 expression, can be explored more thoroughly in the murine system, this laboratory has initiated the isolation of murine cDNA and genomic clones. Two types of murine cDNA clones have been isolated that encode proteins similar to their human counterparts. Surprisingly, however, the two types of clones appear to represent transcripts of different genes, since they differ in nucleotide sequence within regions encoding identical protein segments. This interpretation is consistent with the isolation of two types of genomic clones encoding the Oct-2 POU domain, using the polymerase chain reaction. Genomic analysis and cloning is being pursued rigorously to resolve this structural difference between the murine and human Oct-2 loci.

Dr. Singh is also Assistant Professor of Molecular Genetics and Cell Biology at The University of Chicago.

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DEVELOPMENTAL GENETICS

PHILIPPE SORIANO, PH.D., *Assistant Investigator*

Research in Dr. Soriano's laboratory is focused on early development of the mouse embryo. The two main aspects of this research are 1) the study of cell lineages, during the egg cylinder stages and at gastrulation, and 2) the elucidation of the role of specific genes during development of the embryo, using insertional mutagenesis in transgenic mice. These projects involve both retroviral infection of embryos and embryonic stem (ES) cells and targeted mutagenesis of specific genes by homologous recombination in ES cells. Much of the work on mutagenesis is in close collaboration with Dr. Allan Bradley (Baylor College of Medicine).

I. Retroviruses and Mouse Development.

Classical approaches for the study of lineages, such as injecting dyes into individual cells, are of limited use in the mouse because of rapid dilution of the marker with successive cell divisions. Previous work has shown that genetic mosaicism introduced into developing mouse embryos by retroviruses could be used to obtain information on allocation of cells to specific lineages. These studies demonstrated a high level of cell mixing prior to gastrulation and an early determination of the germline. The aim of the studies that have been initiated over the past year has been to obtain information on cell lineages in the mouse embryo during the egg cylinder stage and at gastrulation.

A retroviral labeling system capable of identifying single cells and their progeny is required to address these issues. The bacterial β-galactosidase gene has been chosen as a reporter gene because of the facility to detect its activity. Because the viral promoter is not active in early embryos, the β-galactosidase gene has to be placed under the control of an internal promoter, in this case the herpes simplex virus thymidine kinase promoter. Retroviral vectors thus expressing the reporter gene have been used to infect both preimplantation and postimplantation embryos.

Because of the block to expression of the viral promoter and because the presence of the viral enhancers has been thought to act as a negative regulatory element in embryonal carcinoma (EC) cells, the effect of the enhancers on the efficiency of expression from internal promoters has been tested in ES cells as a model system. These new retroviral vectors either contain *gag* sequences (Gen vectors), resulting in higher viral titers, or do not extend into the *gag* region (Zen vectors); all vectors include a bacterial *supF* gene to facilitate cloning of the provirus. Studies with these vectors have demonstrated that 1) deletion of the enhancers has no significant effect on viral titer; and 2) expression of the reporter gene is modulated, at the most a few fold, by the presence or absence of the viral enhancers, in both Gen and Zen vectors, and that this effect is the same in fibroblasts, where the viral promoter is active, as in ES cells, where it is not. These results argue against an active repression by viral sequences of expression from an internal promoter in ES cells. The laboratory also has recently generated high titer, viral enhancer, and promoter-minus vectors that are being tested.

II. Retroviral Insertional Mutagenesis.

One approach to the study of the role of genes active in development is their inactivation by germline insertion of foreign DNA in transgenic mice. The approach is attractive, because the foreign DNA serves both as a means to disrupt the gene physically and as a tag for the molecular cloning of the affected gene. Retroviruses appear particularly well suited for this purpose, since they do not cause any rearrangements of flanking sequences and can be engineered to allow for selective cloning of the flanking sequences. Experiments under way in the laboratory involve the introduction of retroviruses into the germline of mice to create new transgenic strains, both by infection of preimplantation embryos and by infection of ES cells. The use of ES cells allows screening of the cells, prior to their reintroduction into blastocysts, and breeding of the chimeras to test for contribution to the germline. The laboratory's first experiments with viral-infected ES cells have demonstrated a significant number of germline chimeras.

To facilitate screening of mutation events, Dr. Soriano and his colleagues have devised an enhancer trap and a gene trap system. In the enhancer trap retroviral construct, lacking the viral enhancers, a β-galactosidase gene, with its initiator codon, has been placed downstream of the viral promoter and of a splice acceptor. The viral vector also contains a *neo* cassette, permitting selection in ES cells, and the *supF* gene to facilitate subsequent cloning. Almost no ES clones infected with this

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virus express β-galactosidase, consistent with previous findings indicating nonactivity of the viral promoter in ES cells; however, differentiation of the stem cells into embryoid bodies results in activation at a high frequency, suggesting expression from the viral promoter. Regional, specific staining of the embryoid bodies is observed, suggesting influence on expression according to the site of integration. This result is surprising, since it had been thought that the block to viral expression is maintained during further development. Gene trap viral vectors, in which the viral promoter has been deleted, have been constructed and are being tested.

III. Targeted Mutagenesis in Transgenic Mice.

ES cells also allow the possibility of selection for mutagenesis of specific genes. Efforts in the laboratory have focused on the gene encoding c-*src*, which has been made available by Dr. David Baltimore (Whitehead Institute). Constructs designed for knockout of c-*src* activity have been introduced into ES cells by electroporation, and homologous recombinant clones have been isolated by screening with the polymerase chain reaction (PCR) technique. With one such construct, including ~8 kb of homology, the frequency of such targeted events is 1 in 100; blot analysis of these clones has demonstrated gene targeting, but also rearrangements of the mutated allele. The laboratory has also mutated the alternate, neuronal splice of c-*src* in ES cells and used both sets of cells for production of chimeras. These animals are being tested for contribution to the germline.

Dr. Soriano is also Assistant Professor of Molecular Genetics and of Cell Biology at Baylor College of Medicine.

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STRUCTURE AND FUNCTION OF SMALL RIBONUCLEOPROTEINS IN MAMMALIAN CELLS

JOAN ARGETSINGER STEITZ, PH.D., *Investigator*

A variety of small ribonucleoproteins (tight complexes between one or more proteins and a short RNA molecule) inhabit all higher eukaryotic cells. Many are highly abundant (more than 10^5 copies/cell) and highly conserved across species, suggesting important roles in cellular metabolism. Different types are localized specifically in the cell nucleus or cytoplasm. Small ribonucleoproteins (RNPs) are often the targets of autoantibodies found in the sera of patients with rheumatic disease. Dr. Steitz's laboratory has been using autoantibody probes to decipher the structure and function of small RNPs in mammalian cells. So far, they all appear to participate in gene expression or genome maintenance. Progress in the past year is summarized according to the category of small RNP.

I. 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 ~6–9 proteins, some unique and some common. These snRNPs belong to the Sm class, as they 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-messenger RNAs of eukaryotic cells.

A. *Use of antibodies directed against yeast splicing proteins to characterize mammalian analogues.* Genetic dissection of the splicing reaction in the yeast *Saccharomyces cerevisiae* has provided insights into spliceosome composition and function. However, such analyses have been largely lacking in the mammalian system. Using antibodies against prp8 (courtesy of Dr. Jean Beggs, University of Edinburgh), a yeast protein essential for splicing and a unique component of the yeast U5 snRNP, Dr. Steitz's laboratory identified an analogue in HeLa cell extracts. Like many other snRNP proteins, this novel 200 kDa protein is also reactive with anti-Sm antibodies. Immunoprecipitation and gradient fractionation analyses have characterized it as a component of the U5 snRNP and of the U4/U5/U6 snRNP complex, which is a precursor to spliceosome assembly. The 200 kDa protein is also present in affinity-purified spliceosomes. Antibodies directed against other yeast splicing proteins (courtesy of Dr. John Abelson, California Institute of Technology) are being exploited to identify additional HeLa proteins implicated in splicing.

B. *Dissecting the mechanism of trans-splicing.* The trans-splicing reaction occurring in trypanosomes and related species (*Trypanosoma brucei*, *T. cruzi*, *T. vivax*, *Leishmania enriettii*, *Leptomonas collosoma*, *Crithidia fasciculata*), as well as in the nematode *Caenorhabditis elegans*, involves the transfer of a 5′ exon from a spliced leader transcript (SL RNA) onto a pre-mRNA transcript possessing a 3′ splice acceptor. Dr. Steitz's laboratory previously discovered that SL RNAs assemble together with proteins precipitable by anti-Sm antibodies. Thus SL RNAs seem to fulfill a dual function in the trans-splicing process—that of both the 5′ exon and an snRNP RNA. To test the prediction that the SL snRNP might autonomously activate its own 5′ splice site and thereby eliminate the need for a U1-like snRNP in the trans-splicing machinery, splicing substrates that contain an SL RNA sequence (from *L. collosoma*) connected to a 3′ splice site (from the adenovirus major late-transcription unit) have been constructed. Such constructs splice well in HeLa cell nuclear extracts. Next, U1 snRNPs were inactivated in the extract by extensive oligonucleotide-directed RNase H degradation of the 5′ end of U1. Splicing of a control transcript containing intron 1 of adenovirus was completely abolished; however, the SL RNA–3′ splice-site construct retained substantial activity. Thus the SL RNA sequence does appear to substitute for U1 during splicing in HeLa cell nuclear extracts. Studies employing mutant SL RNA–3′ splice-site constructs are currently in progress to define the precise sequence requirements for U1 snRNP-independent splicing. These results underscore the close relationship of the cis- and trans-splicing machineries and suggest that trans-splicing could exist in mammalian cells.

II. 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) that also have 5′ trimethylguanosine on their RNAs and contain proteins reactive with anti-Sm autoantibodies. Two of the RNAs in such low abundance snRNPs (U11 and U12)

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have been sequenced, and others are now being analyzed. Functions for most minor snRNPs are still unknown, although the U11 snRNP has been implicated in the polyadenylation reaction that fashions the 3′ ends of most eukaryotic messenger RNAs.

A. *Role of the human U7 snRNP in the 3′-end maturation of histone pre-mRNAs.* One low-abundance snRNP, containing U7 RNA, functions in the 3′-end processing of histone pre-mRNAs, in which an endonucleolytic cut is made between two conserved signals in the pre-mRNA. The two conserved signals are recognized by distinct components of active nuclear extracts: the upstream stem loop associates with a nuclease-insensitive factor, while binding to the downstream element is mediated by the snRNP, presumably via base pairing with the 5′ end of the U7 RNA. Accordingly, the laboratory recently established that pre-mRNA mutations in the stem loop only reduce processing, whereas deletion of the downstream element abolishes activity. To delineate the exact sequence requirements for the interaction between the human U7 snRNP and the downstream conserved element, Dr. Steitz and her colleagues are pursuing *in vitro* reconstitution of active U7 snRNPs. Vectors capable of *in vivo* expression of marked and/or mutated U7 snRNPs have also been developed and are being used in suppression studies with mutant histone genes.

B. *Structure and function of* Herpesvirus saimiri–*encoded U RNAs.* It was previously established that marmoset T lymphocytes transformed by *Herpesvirus saimiri* contain four novel virus-encoded U RNAs [*Herpesvirus saimiri* U RNAs (HSURs)]. HSURs assemble with Sm proteins and acquire a 5′ trimethylguanosine cap, categorizing them as typical Sm snRNPs (of low abundance). Dr. Steitz's laboratory has now discovered a fifth HSUR, encoded by a nearby region of the viral genome; HSUR 5 is related (but not identical) to HSURs 1 and 2. These three HSURs share 5′-end sequences that exhibit both complementarity to the AAUAAA polyadenylation signal and homology to AU-rich sequences found in the 3′ untranslated regions of short-lived mRNAs for certain lymphokines, cytokines, and proto-oncogenes. Therefore the possibility that HSURs contribute to cell transformation either by acting in mRNA polyadenylation or by inhibiting the selective degradation of (and thereby stabilizing) important cellular messengers is being pursued.

III. Other snRNPs.

A. *Analyses of the 7SK RNP.* The 7SK RNP is an abundant (2×10^5/cell) nuclear particle of unknown function in mammalian cells. The secondary structure of the 7SK RNA alone and within its RNP have been analyzed by chemical modification: base-specific reagents are used to modify selectively single-stranded nucleotides, which are then mapped by primer extension. Based on these results, oligonucleotides complementary to single-stranded regions have been identified that can degrade the 7SK RNA with RNase H in cell extracts to test involvement in various *in vitro* RNA-processing reactions. Currently, hints that the 7SK RNP may be an auxiliary factor in splicing that transiently associates with the U4 and/or the U6 snRNP are being pursued.

B. *New nucleolar snRNPs.* Autoantibodies previously known to precipitate the U3 RNP (a conserved, abundant nucleolar snRNP) have been used to isolate and characterize two additional small nucleolar RNAs from HeLa cells. Both RNAs possess a trimethylguanosine cap structure but are not precipitable by anti-Sm antibodies. Sequence analysis has revealed that one of these RNAs (136 nucleotides long) is 85% homologous to the rodent U8 RNA. It is therefore referred to as human U8, while the other RNA (105 nucleotides) represents a novel species, U13. Human U8 and U13 are present at ~20% and 5% the amount of U3 (10^6/cell), respectively. Both contain two conserved sequences (boxes C and D), which are also present in human U3 RNA and in U3 RNAs from unrelated organisms. The role of box C and/or D in binding the common 34 kDa autoantigen (otherwise known as fibrillarin) is under study. The U3, U8, and U13 RNPs appear to comprise a new subset of mammalian snRNPs, whose roles in ribosome biogenesis are being investigated.

C. *The human telomere transferase enzyme is an snRNP that synthesizes TTAGGG repeats.* The large number of chromosomes (10^4–10^7) generated by some ciliates has provided an advantageous system for the study of the structure, function, and biosynthesis of telomeres. Telomere terminal transferase (telomerase), first isolated from *Tetrahymena*, surprisingly turned out to be a ribonucleoprotein that synthesizes telomeres *de novo*.

Despite the fear that telomerase activity might be negligible in mammalian cells (~50 chromosomes),

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Dr. Steitz's laboratory has identified this activity in crude extracts of HeLa cells. When the extracts are incubated with $MgCl_2$, dATP, TTP, dGTP, and the synthetic oligonucleotide $(TTAGGG)_4$ (representing the human telomere sequence), DNA products that form a 6 nucleotide repeating pattern are observed. Oligonucleotides containing nontelomeric sequences are not primers for the reaction, whereas oligonucleotides representing telomeres of other organisms can replace the $(TTAGGG)_4$ primer, giving synthesis of the identical TTAGGG repeat sequence. Telomerase activity is sensitive to ribonuclease A, micrococcal nuclease, and proteinase K, indicating the enzyme is probably a ribonucleoprotein. A search for the telomerase RNA component is currently under way. This is the first demonstration of telomerase activity in higher eukaryotes and establishes the generality of telomerase-mediated telomere replication.

IV. Transcription and Function of Epstein-Barr Virus Small RNAs.

Lymphocytes infected with Epstein-Barr virus (EBV) produce two virus-encoded small nuclear RNAs, EBER 1 and 2. Their high abundance (up to 10^7/cell) in latency suggests that EBERs are involved in EBV-directed cell immortalization, but their exact function is not known. Small RNAs similar to EBERs exist in cells infected with EBV-like viruses of baboons and chimpanzees.

Previously, Dr. Steitz's laboratory discovered that EBER genes are unique in having active promoter elements that resemble those typical of both class II and class III genes. In addition to internal control regions, they have upstream sites that bind the Sp1 transcription factor and activating transcription factor (ATF), as well as a TATA box. Presently the laboratory is asking how RNA polymerase II is prevented from transcribing the EBER genes, focusing attention on the TATA box. Whether the EBERs are transactivated by a virally encoded protein or induced by cAMP, which is known to function through the ATF site, is also being investigated.

Studies of EBER function have begun with an analysis of associated proteins. The only protein known to bind EBERs is the La autoantigen, a 50 kDa phosphoprotein that associates at least transiently with the 3′ ends of all RNA polymerase III transcripts and has been implicated in transcription termination. Recently a second novel protein has been identified and is being cloned. Its characterization should help elucidate the role of EBER in the transformed lymphocyte.

Dr. Steitz is also Professor of Molecular Biophysics and Biochemistry at Yale University School of Medicine.

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GENES RESPONSIVE TO GROWTH FACTORS

VIKAS P. SUKHATME, M.D., PH.D., *Assistant Investigator*

The primary focus of Dr. Sukhatme's laboratory is on characterizing genes that are growth factor inducible and encode transcription factors. The expectation is that genes of this type will play broad roles as "nuclear transducers" by coupling early biochemical events that follow within seconds of ligand-receptor binding to long-term cellular responses required for proliferation and differentiation. Until recently, c-*fos* was the only example of such a gene. Now work from several laboratories indicates that there are at least three other sets of early response genes that are likely to encode transcription factors: *Egr-1*, *-2*, and *-3*; c-*jun* and *junB*; and *nur77*. The Egr family discovered in Dr. Sukhatme's laboratory are zinc finger proteins of the Cys_2-His_2 class. The long-term goal of these studies is to understand the role of these gene products in cellular proliferation, as well as in signal transduction and cellular differentiation.

I. *Egr-1*.

A. *Is* Egr-1 *induced in every mitogenic process?* Earlier work from Dr. Sukhatme's laboratory identified a cDNA designated *Egr-1*, also referred to as NGFI-A, *zif268*, and Krox24, respectively, by the groups of Drs. J. Milbrandt (Washington University), Daniel Nathans (HHMI, The Johns Hopkins University), and R. Bravo (European Molecular Biology Laboratory, Heidelberg), who have discovered the same gene independently. This transcript was growth factor inducible in diverse cell types by various mitogens. To date, *Egr-1* induction has been noted upon mitogenic stimulation of murine and human fibroblasts, rat liver H35 hepatoma cells, rat renal mesangial and glomerular cells, monkey kidney BSC-1 cells, murine splenic B cells and T cell clones, and human peripheral blood lymphocytes, as well as in liver regeneration. Different mitogens, including many cell-type-specific ones (e.g., anti-μ antibody for B cells), were used. These data show the ubiquitous nature of *Egr-1* induction during the onset of growth. Dr. Sukhatme's laboratory is currently asking whether there is a causal relationship; i.e., is *Egr-1* stimulation sufficient and/or necessary for mitogenesis? Is *Egr-1* a proto-oncogene?

B. *What changes in a cell's environment lead to* Egr-1 *induction? What intracellular pathways are used? Why is* Egr-1 *often co-regulated with* c-fos? The emphasis here is on understanding the events—from cell surface to nucleus—that stimulate *Egr-1*. Diverse mitogens induce *Egr-1* mRNA. Because these mitogens act through different second messenger systems, it appears that *Egr-1* stimulation lies at the convergence of these various pathways. Furthermore, since these second messengers are generated by processes unrelated to cell growth, *Egr-1* modulation may be more generally coupled to changes in a cell's extracellular environment, e.g., by ionic changes, hormones, or neurotransmitters. Dr. Sukhatme's previous work had shown that *Egr-1* is a nerve growth factor–inducible gene in rat PC12 cells, and cellular depolarization *in vitro* and seizure activity *in vivo* can induce its expression. During the last year these observations have been extended to other ligand-receptor interactions. In collaboration with Dr. K. Chien (University of California at San Diego), it has been found that both α- and β-adrenergic stimulation of cardiac myocytes causes striking *Egr-1* expression, as does renal ischemia [collaboration with Dr. J. Bonventre (Harvard Medical School)]. Observations such as these are being extended to other systems so as to define the broad range of proximal events—from extracellular ligand-receptor interactions to intracellular second messengers—that modulate *Egr-1* expression.

With regard to the latter, initial studies in Dr. Sukhatme's laboratory have been in fibroblasts. Addition of the phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate (TPA) to these cells induces *Egr-1* mRNA. However, even in cells in which protein kinase C (PKC) has been downregulated, *Egr-1* can be induced by epidermal growth factor (EGF) and by serum. Thus multiple intracellular pathways in fibroblasts converge to induce *Egr-1*. To define the cis-acting elements that mediate these inductions, Dr. Sukhatme's laboratory recently cloned and sequenced the *Egr-1* gene, identified the transcription initiation site, and sequenced ~1 kb of upstream sequence. Of note are several putative cAMP response elements and AP1-binding sites as well as six so-called CArG boxes that form the inner core of the *fos* serum response element (SRE). Chloramphenicol acetyltransferase (CAT) assays in fibroblasts utilizing deletion mutants of the 5′ regulatory region suggest that these elements are responsible both for the TPA induction and serum inducibility of *Egr-1*. Gel shift assays show that

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these elements bind to a factor present in fibroblasts as well as HeLa cell nuclear extracts and that this binding is competed for by the *fos* SRE. Thus it is likely that the serum response factor (SRF) that binds to the *fos* SRE mediates both serum and TPA inducibility of *Egr-1*. Evidence has emerged from studies using a cDNA clone for SRF that show that the *in vitro*–generated SRF protein binds to the *Egr-1* upstream CArG boxes. However, this analysis does not exclude the possibility that other SRF-like proteins exist. Experiments intended to explore this hypothesis are in progress.

With the *Egr-1* 5′ region deletion mutants, Dr. Sukhatme's laboratory is also defining how *Egr-1* induction is mediated in the many non-growth-related situations mentioned above. Attempts to define the mechanisms by which *Egr-1* transcription is negatively regulated are also under way.

C. *Properties of the Egr-1 protein.* During the last year, antisera against an Egr-1–specific peptide and a fusion protein have been generated. Both antisera give intense nuclear staining of serum-stimulated fibroblasts. Peak signal intensity is noted between 2 and 3 hours, but the signal persists for at least 5 hours. Western analysis and immunoprecipitation show an 80 kDa band in the serum-induced fibroblasts, a size in agreement with that seen for the *in vitro*–translated product. The Egr-1 protein is also rapidly phosphorylated, suggesting that this mechanism may be an additional means of modulating its activity.

D. *Developmental expression of* Egr-1 *shows coregulation with* c-fos. [These studies were conducted in collaboration with A. McMahon (Roche Institute of Molecular Biology).] *Egr-1* expression is easily seen in total embryonic RNA by day 11. *In situ* hybridization shows striking localization to the perichondral regions of long bones and digits, to mesenchymal odontoblasts, and to roots of hair whiskers, and intense but patchy signal in various muscle groups. Several inferences may be derived from these developmental expression patterns: 1) The bone and tooth data are similar to those obtained for c-*fos* and point to the rather special relationship between these two genes. 2) Possible targets for Egr-1 action might include collagen type I, since a major function of odontoblasts and osteoblasts is to secrete collagen. 3) Nerve growth factor (NGF) receptors have recently been localized to several nonneuronal tissues, including the dental ridge, muscle, and limb buds. Thus *Egr-1* mRNA induction may be mediated by NGF in these tissues. 4) Osteoblasts or cell lines derived from them might be a particularly interesting system in which to study *Egr-1* (and c-*fos*) expression with an eye to asking what keeps *Egr-1* expression at constitutively high levels in these cells. Findings in these cells could be contrasted to those derived from serum-stimulated fibroblasts, where expression of these genes is transient.

E. EGR1 *expression in human tumors.* To analyze whether *EGR1* is deregulated in abnormal cell growth, Northern blots of human tumors were provided to us by Dr. D. Slamon (University of California at Los Angeles). In over 50% of the cases, *EGR1* expression is reduced 3- to 10-fold in tumor tissue versus surrounding normal tissue from the resected human specimens. The tumors were derived from lung, colon, bladder, liver, breast, bone, and muscle. In the near future, Dr. Sukhatme and his colleagues will look at whether these data can be substantiated at the protein level. What might decreased tumor expression imply? One possibility is that EGR1 functions as part of a negative growth regulatory pathway in a manner similar to the retinoblastoma gene product. Earlier data from Dr. Sukhatme's laboratory on the $5q^-$ deletions in therapy-related acute nonlymphocytic leukemia (ANLL) that map to the same regions as the chromosomal localization of *EGR1* would tend to support this view. Clearly, attempts at overexpressing and/or inhibiting *EGR1* expression in both normal and transformed cells will be needed to answer these questions.

II. Genes Related to *Egr-1*.

During the past year, Dr. Sukhatme's laboratory has used the finger domain probe of *Egr-1* to rescreen the original serum plus cycloheximide fibroblast cDNA library. Three novel genes (*Egr-2*, *-3*, and *-4*) have been partially characterized. All share nearly identical DNA-binding domains but differ elsewhere in their sequence. *Egr-2* [also known as Krox20; Drs. R. Bravo and P. Charnay (European Molecular Biology Laboratory, Heidelberg)] and *Egr-3* are both novel immediate-early response genes. Their transcripts [~3.2 kb (*Egr-2*) and ~5 kb (*Egr-3*)] show kinetics similar to those of *Egr-1*. The nonfinger regions of *Egr-2* and *Egr-3* appear to differ substantially from those of *Egr-1*. Studies along the lines described above for *Egr-1* are under way for these two genes as well.

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Dr. Sukhatme is also Associate Professor of Medicine and of Molecular Genetics and Cell Biology and Member of the Committee on Immunology at The University of Chicago.

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CELLULAR MEMBRANE RECEPTOR SIGNALS AND THE HEPATIC GROWTH RESPONSE

REBECCA A. TAUB, M.D., *Associate Investigator*

The major interests of Dr. Taub's laboratory are 1) characterization of the growth-response genes of hepatic cells, 2) analysis of insulin receptor gene expression, and 3) studies of receptor-ligand interactions through analyses of antireceptor monoclonal antibodies.

I. Characterization of the Growth-Response Genes of Hepatic Cells.

The liver is composed primarily of hepatocytes, cells of epithelial cell origin, that perform vital metabolic and synthetic functions. Although the adult liver does not normally proliferate, it retains the capacity to regenerate after partial hepatectomy or chemical injury. This regeneration appears to be accomplished by the induction of DNA synthesis in virtually all of the remaining hepatic cells. Once the liver has regained its initial size, it reenters a quiescent state. The growth factors, both circulating and local, involved in this tightly regulated process may include insulin, glucagon, epidermal growth factor (EGF) [or transforming growth factor α (TGF-α)], TGF-β, and other putative hepatic growth factors. Dr. Taub's laboratory is approaching the complex series of early events leading to liver regeneration after partial hepatectomy in several ways. After identifying the immediate-early hepatic growth-response genes, the laboratory will be able to compare them with those genes previously shown to be involved in growth responses in other tissues, such as fibroblasts and lymphocytes. In fibroblasts and lymphocytes, the immediate growth-response gene products appear to fall into two large classes: 1) secreted proteins that may function in autocrine growth regulation and 2) nuclear proteins that may regulate subsequent nuclear events. Although it is presumed that growth and autocrine factors vary significantly in different tissues, it is not yet known if the same pattern of expression of nuclear regulatory proteins is common to most growing tissues. The long-term goal of this research is to understand the genetic regulation of growth in hepatic and other epithelial cells.

The laboratory uses two liver cell systems in which to study hepatic growth. The first is an H35 rat hepatoma cell line that becomes quiescent under serum-deprived conditions and initiates DNA synthesis in response to insulin. In this cell line, physiologic concentrations of insulin stimulate many of the immediate-early growth-response genes such as *myc*, *fos*, *egr-1* and *egr-2*, and *jun* that are stimulated in mitogen-treated fibroblasts and regenerating liver. Because it is a cell line, growth conditions can be easily manipulated. However, this H35 cell line does not possess all of the normal hepatic properties, and therefore regenerating rat liver itself is a second liver cell system used to identify hepatic growth-response genes.

The techniques of differential screening and cDNA subtraction analysis have yielded exciting results in identifying immediate-early growth-response genes in fibroblasts and lymphocytes. These techniques were applied to regenerating liver cDNA libraries prepared 3 h post-hepatectomy in the presence of cycloheximide, and ~20 unique induced genes were isolated. A similar number of genes has been isolated from H35 cDNA libraries prepared from cells treated for 3 h with insulin in the presence of cycloheximide. Several criteria are used to identify an induced gene of particular interest: 1) The gene must be different from previously identified genes (such as *myc*, *fos*, *egr*, *jun*), as determined by hybridization studies and DNA sequence analyses. 2) On genomic Southern analysis, the gene should be unique or a member of a gene family, and conserved across species. 3) Preferably the gene is specifically or predominantly expressed in regenerating liver (and H35 cells) and not in mitogen-treated fibroblasts. However, some genes appear to be expressed specifically in regenerating liver and not H35 cells.

Studies are continuing to characterize the complete immediate-early genomic response in liver regeneration and to perform a more detailed analysis of specific induced hepatic genes.

II. Regulation of Expression of the Human Insulin Receptor Gene.

The insulin receptor (IR) is an essential protein present on the surface of virtually all cells. Little is known about the control of the level of this protein on cellular surfaces, but it has been found that the IR level correlates roughly with the level of IR gene transcripts within cells. The IR gene promoter is like other housekeeping promoters: it lacks a TATA box and has multiple transcriptional initiation sites, primarily within a 300 bp GC-rich region. The long-term goal of this project is to understand the rela-

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tive roles of transcriptional and post-transcriptional regulation of the IR gene and how this regulation compares with other housekeeping genes.

Reporter gene analysis using human IR promoter–chloramphenicol acetyltransferase (hIR-CAT) fusion plasmids established regions responsible for promoter activity and verified the localization of the major IR gene transcriptional initiation sites. However, transfection with hIR-CAT plasmids containing upstream regions resulted in increased utilization of the most 5′ IR gene mRNA initiation sites in transfected relative to untransfected cells. Reporter gene analysis also established that a region of the IR promoter and first exon containing all of the transcriptional initiation sites is more active in HepG2 than CV1 cells. Because the steady-state level of expression of the IR gene is much higher in HepG2 than CV1 cells, the results of the reporter gene analysis may reflect tissue-specific differences in IR gene transcription. Such tissue-specific transcriptional regulation would be a novel finding in a housekeeping promoter. Unlike the reporter gene analyses, nuclear run-on studies of IR mRNA give similar results in HepG2 and CV1 cells, suggesting that IR gene expression may not be regulated in a tissue-specific manner at the level of RNA initiation. The different conclusions drawn from reporter gene and nuclear run-on analyses could be explained if downstream GC-rich regions within the IR first exon and intron, not included in the reporter gene constructions, contribute significantly to the basal activity of the IR promoter, thus eliminating the tissue-specific transcriptional effect mediated by upstream sequences.

In addition, it appears that cells from a patient with severe insulin resistance and markedly reduced levels of IR mRNA give normal results in nuclear run-on studies of the IR gene. The IR gene mutation in this patient may cause a post-transcriptional effect on IR gene stability.

III. Antireceptor Monoclonal Antibodies That Bind to the Ligand-binding Domain.

Dr. Taub's laboratory previously noted that a linear sequence, arginine-tyrosine-aspartic acid, within the hypervariable region of an antifibrinogen receptor monoclonal antibody, PAC1, appeared to mimic the arginine-glycine-aspartic acid sequence within fibrinogen that recognizes the fibrinogen receptor. These studies are being extended by isolating anti-idiotypic antibodies directed against the PAC1 monoclonal antibody and peptides within the PAC1 variable regions to determine if these anti-idiotypic antibodies have linear amino acid sequences similar to the fibrinogen receptor. In addition, the PAC1 monoclonal antibody variable regions are being expressed in a prokaryotic system to allow further genetic manipulation of the sequences important in fibrinogen receptor binding.

Further studies are under way to determine if this immunologic approach to receptor-ligand interactions may be generally applied to other antireceptor monoclonal antibodies. Dr. Taub and her colleagues are presently analyzing anti-idiotypic antibodies isolated by Dr. Bernard Erlanger (Columbia University), directed against the thyroid-stimulating hormone (TSH) receptor, that appear to bind to the TSH-binding site. The analyses of these anti-TSH receptor monoclonal antibodies could prove particularly interesting, because in Graves' disease, autoantibodies to the TSH receptor may also bind to the TSH-binding site on the TSH receptor. The immunoglobulin variable regions from anti-TSH receptor anti-idiotypic antibodies are presently being sequenced and the sequences analyzed for similarity with TSH.

Dr. Taub is also Assistant Professor of Human Genetics at the University of Pennsylvania School of Medicine.

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MOLECULAR BASIS OF METAMORPHOSIS IN *DROSOPHILA*

CARL S. THUMMEL, PH.D., *Assistant Investigator*

Dr. Thummel's laboratory is studying the molecular basis of metamorphosis in *Drosophila melanogaster*. Metamorphosis is triggered at the end of larval development by an increase in the titer of the steroid hormone ecdysone. In response to this signal, the imaginal tissues grow and differentiate to form the adult fly, while most larval tissues are histolyzed.

The ecdysone–receptor protein complex controls these developmental changes by triggering a complex genetic cascade. The effect of ecdysone on gene expression can be visualized by observing changes in the puffing pattern of the giant polytene chromosomes in the larval salivary glands. A detailed study of this response has been carried out by Michael Ashburner and his colleagues. The hormone directly induces a small set of early puffs. One or more of these genes are required for repression of the early genes and induction of a large set of more than 100 late genes. The late genes are thought to play a direct role in initiating metamorphosis. By isolating and characterizing the early ecdysone-inducible genes and determining the mechanism of their induction, 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 gene hierarchies are controlled during development.

Current work in Dr. Thummel's laboratory is focused on an early ecdysone-inducible gene, *E74*, which is located at position 74EF in the polytene chromosomes, within one of the largest early puffs described by Ashburner. This gene was isolated in collaboration with Ken Burtis and C. Weldon Jones in the laboratory of Dr. David Hogness at Stanford University.

E74 consists of three transcription units that arise from unique promoters and share a common polyadenylation site. The *E74A* promoter drives the synthesis of a 60 kb primary transcript that is spliced to form a 6 kb mRNA. Two internal promoters, located 40 kb downstream from the *E74A* promoter, direct the synthesis of the *E74B* mRNAs, 4.7 and 5.0 kb in length. Similar complex giant genes have been found in *Drosophila*, although only in regulatory genes that are known to play a central role in development, such as the homeotic genes *Antennapedia* and *Ultrabithorax*. Transcription of the *E74A* unit is induced several orders of magnitude as a direct response to ecdysone. Furthermore, the temporal profile of *E74A* mRNA accumulation parallels the 74EF puffing response in the polytene chromosomes. *E74B* transcription, on the other hand, does not appear to be induced by ecdysone directly, although these RNAs are present at some of the same times during development as the *E74A* transcript.

The *E74A* and *E74B* transcripts each contain unique 5′ exons joined to a common set of three 3′ exons. This sequence arrangement leads to the synthesis of two related *E74* proteins, each with a unique amino-terminal domain joined to a common carboxyl-terminal domain. The amino-terminal domains of the *E74* proteins contain regions rich in acidic amino acids, whereas the carboxyl terminus is rich in basic amino acids. These domains are separated by a "spacer" region of repetitive amino acids. The apposition of separate acidic and basic domains is reminiscent of yeast transcriptional activators. Comparison of the *E74* proteins to the protein sequence database revealed that 84 amino acids near the carboxyl terminus are 50% identical to the protein encoded by the *ets*-2 proto-oncogene. The function of *ets*-2 is unknown, although its activity is correlated with cellular proliferation during mouse development and *ets*-2 protein is localized in the nucleus.

Dr. Thummel's laboratory has begun to characterize the biochemical properties of the *E74A* protein. Protein synthesized *in vitro* was used to scan a 100 kb region encompassing the *E74* gene for potential binding sites. A single site, 30 kb downstream from the *E74A* promoter, was identified. This fragment was also bound by *E74A* protein that was prepared through overproduction in bacteria and found to contain two binding sites, as determined by DNase I footprinting. Antibodies directed against the *E74A* protein stain salivary gland nuclei and reveal specific binding sites in the polytene chromosomes, indicating that *E74A* protein also binds DNA *in vivo*. The polytene chromosome stains should allow the identification of genes that are potentially regulated by *E74A* and thus direct future studies to the next level in the regulatory hierarchy.

Ecdysone is a transducer of temporal information in *Drosophila* development. The precise timing of gene expression in response to ecdysone is evident

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in the highly reproducible pattern of puffs that arise in the polytene chromosomes. Much of Dr. Thummel's research is focused on characterizing the temporal control of *E74* expression, in an effort to understand how the timing of gene expression is regulated during development. The timing of *E74A* expression appears to be controlled at both transcriptional and translational levels.

Ecdysone induces synthesis of the *E74A* mRNA at the level of transcriptional initiation, in a manner similar to glucocorticoids. RNA polymerase moves along the *E74* gene at a rate of 1.1 ± 0.3 kb/min, in agreement with the *in vivo* transcription rate described by others. Furthermore, as expected from the length of the gene, cytoplasmic *E74A* mRNA can first be detected 60 min after the addition of ecdysone. This supports a model that gene length in *Drosophila* may provide a way to control the time between the inducer signal (ecdysone) and the product (*E74A* mRNA). The observation that *E74A* mRNA appears in the cytoplasm shortly after transcription termination suggests that processing of the primary transcript is not rate limiting. This has been confirmed by RNase protection assays, which reveal splicing of nascent *E74A* transcripts prior to polyadenylation.

The appearance of *E74A* protein may be further delayed at the post-transcriptional level. The *E74A* mRNA contains an unusually long 5′ leader, 1,891 nucleotides in length, with 11 short open reading frames. This leader may function to regulate translation of the *E74A* transcript. Initial Western blotting experiments indicate that *E74A* protein is present at high levels at the white prepupal stage, several hours after the mRNA begins to accumulate to high levels. This delay in expression may help confer tissue specificity to the expression of *E74A*. High levels of *E74A* mRNA can be detected by *in situ* hybridization in virtually all late larval tissues. By the white prepupal stage, however, the RNA distribution is more restricted, with high levels in the central nervous system and imaginal disks. It will be interesting to see if this pattern correlates with the pattern of *E74A* protein accumulation. Mutational analysis of the 5′ leader should indicate whether these sequences exert a regulatory role in *E74A* protein synthesis.

Dr. Thummel is also Assistant Professor of Human Genetics at the University of Utah School of Medicine.

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DEVELOPMENTAL REGULATION OF GENES

SHIRLEY M. TILGHMAN, PH.D., *Investigator*

I. Transcriptional Control of the α-Fetoprotein–Albumin Locus.

This laboratory is investigating genetic and molecular mechanisms that govern cell type specification during embryogenesis in the mouse. One approach to this problem has been to study in detail the transcriptional activation of the mouse α-fetoprotein (AFP) and albumin genes; the expression of these genes is restricted to a subset of endodermal cells in the mouse embryo. Molecular genetic approaches have been used to identify the DNA regulatory signals that are required for their activation, and a combination of genetic and biochemical approaches have been applied to the study of the proteins that interact with those signals.

A. *Identifying DNA regulatory elements.* The albumin and AFP genes constitute a small, linked gene family on mouse chromosome 5. Studies in many laboratories had delineated the positions of two sets of transcriptional regulatory elements surrounding the genes. Their roles in development were assessed by introducing segments of the locus into the mouse germline by microinjection of zygotes. These experiments demonstrated that three enhancers that lie between the two genes are essential for the activation of both genes early in development in liver and that a fourth enhancer 5′ to the albumin gene exhibited little activity before birth. This dependence of both genes on the same regulatory signals provides an explanation for the maintenance of their linkage, since they arose from a duplication approximately 500 million years ago.

Although the genes are co-activated in three cell types—the fetal liver, gut, and visceral endoderm of the yolk sac—the neonatal liver continues to transcribe the albumin gene at a high rate while it represses transcription of AFP. The element responsible for the selective repression of the AFP gene in neonatal liver was localized to a small segment of DNA, between the enhancers and promoter of the gene, that acts as a dominant silencer sequence. Its removal from the gene results in its constitutive expression in adult liver, indicating that the positive trans-acting factors necessary for AFP transcription are present in adult liver.

Removal of the silencer has a less profound effect on the repression of the AFP gene in the adult gut. *In situ* hybridization studies have shown that AFP mRNA is synthesized in all of the epithelial cells lining the rudimentary villi in the fetal gut. AFP mRNA in the adult gut, however, is restricted to a small number of enteroendocrine cells, which represent ~1% of the cells of the adult villi. Thus in the adult gut the stem cells of the crypts are continuously generating two populations of mature cells, the majority of which have the AFP gene in a silent configuration and a small minority of enteroendocrine cells that transcribe the gene at a high rate. The repression in the majority of cells must proceed through a mechanism that is not identical to that in the adult liver, as the silencer defined for liver does not appear to play such a central role in the gut.

B. *Trans-acting factors that interact with the AFP gene.* The tissue-specific behavior of the AFP gene promoter has been attributed to the action of an endoderm-specific factor, HNF-1. Its importance in AFP transcription was demonstrated by generating specific mutations in one of the two HNF-1–binding sites of the gene. This mutation has a deleterious effect on transcription in both liver and gut-derived cell lines.

Genetic analysis in inbred strains of mice identified a locus, *raf*, that encodes a liver-specific cell-autonomous function that is necessary for the postnatal repression of the AFP gene. By crossing transgenic mice carrying various mutations in the AFP gene silencer with BALB/cJ mice, which harbor a mutation in the *raf* gene, Dr. Tilghman's laboratory showed that the *raf* gene product does not interact with the silencer but requires one or more elements in the promoter or gene body itself.

II. Unusual Properties of the H19 Gene.

The mouse H19 gene was originally identified in a screen of fetal-specific liver cDNAs for genes that were under the regulation of *raf*. DNA sequencing revealed that the longest open reading frame in the gene was contained entirely within the first of its five exons, sufficient to encode a protein of only 14 kDa. The likelihood that this reading frame was used *in vivo* was reduced when the sequence of the human homologue was determined. Like the mouse gene, no long open reading frame was present. More striking, no open reading frame was held in common with the mouse gene, despite the fact

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that long tracts of both genes are more than 80% similar. The RNA product of the H19 gene was not found associated with the translational machinery of the cell, but rather in an mRNP particle, reinforcing the conclusion that the RNA is not a classical mRNA.

In situ hybridization studies have shown that the RNA product of the H19 gene is present at high levels in the majority of endoderm and mesoderm cell types in the mouse embryo but is completely absent from all ectodermal derivatives. By introducing a mutated version of the H19 gene into the mouse germline, the laboratory has demonstrated that the DNA elements necessary for the endoderm and mesoderm expression can be functionally separated. Two endoderm-specific enhancers lie 8 kb pairs of DNA 3′ to the gene itself. The elements responsible for mesoderm expression lie outside this domain.

III. Analysis of Large Segments of the Mouse Genome.

The laboratory is constructing a permanent library of the mouse genome in yeast artificial chromosomes. The long-term goal of this project is to facilitate the mapping and isolation of genes for which interesting developmental mutations exist, but which have not yielded to conventional molecular genetic techniques. A second goal is to use the large segments of DNA as substrates for efficient homologous recombination into the mouse germline.

Dr. Tilghman is also Howard A. Prior Professor of the Life Sciences in the 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*

This past year, Dr. Tjian's efforts have been directed at the cloning and characterization of genes encoding eukaryotic DNA-binding transcription factors. These studies have defined novel protein structural domains responsible for DNA-binding specificity, protein dimerization, and transcriptional activation. In addition, Dr. Tjian's experiments have begun to address the important issue of mechanisms that operate to confer promoter-selective gene activation in animal cells. Finally, a number of unexpected findings have opened the way to defining new regulatory factors involved in *Drosophila* gene control during embryonic development and tissue-specific expression in the nervous system.

I. Mechanism of Action of Transcription Factor Sp1.

The SV40 early promoter, which contains six Sp1-binding sites (GC boxes), is induced up to 500-fold in cells expressing Sp1, whereas promoters with fewer sites are activated less efficiently. Analysis of Sp1 mutants reveals multiple distinct regions outside of the DNA-binding domain that are responsible for mediating transcriptional activation. The two most active domains, which appear to be functionally redundant, consist of an unusual structure with a very low charge density but a strikingly high glutamine content. A number of other sequence-specific transcription factors, such as the *Drosophila zeste* protein and several homeodomain proteins, contain glutamine-rich stretches. Dr. Tjian proposes that these glutamine-rich domains represent a novel structural motif for transcriptional activation.

The regulation of gene expression depends, in part, on interactions between sequence-specific transcription factors. Dr. Tjian has been examining the role of protein-protein interactions in modulating the activity of Sp1, a human transcription factor that utilizes glutamine-rich activation domains. These glutamine-rich regions may represent a commonly used structural motif, since a nonhomologous glutamine-rich segment from the *Drosophila Antennapedia* protein is also a potent activator when fused to the Sp1 DNA-binding domain. Sp1 is generally considered a proximal promoter factor that can only stimulate transcription when bound close to the transcriptional start site. However, Dr. Tjian has obtained evidence that distally and proximally bound molecules of Sp1 can stimulate transcription synergistically. In addition, a DNA-binding-deficient mutant of Sp1 that retains active glutamine-rich domains can interact productively with proximally bound Sp1 to superactivate transcription. Glutaraldehyde crosslinking provides direct evidence for a protein-protein interaction between Sp1 monomers. These findings provide evidence for an Sp1:Sp1 interaction that may play an important role in Sp1-mediated transcriptional activation.

II. Structure and Function of the Jun/AP-1 Family of Enhancer Factors.

The discovery by Dr. Tjian and his colleagues that the activator protein-1 (AP-1) family of enhancer binding factors includes a complex of the cellular Fos (c-Fos) and cellular Jun (c-Jun) proteins established a direct and important link between oncogenesis and transcriptional regulation. Recently Dr. Tjian showed that homodimeric c-Jun protein synthesized *in vitro* is capable of binding selectively to AP-1 recognition sites, whereas the c-Fos polypeptide is not. When cotranslated, the c-Fos and c-Jun proteins can form a stable, heterodimeric complex with the DNA-binding properties of AP-1/c-Jun. The related proteins JunB and v-Jun are also able to form DNA-binding complexes with c-Fos. Directed mutagenesis of the c-Fos protein reveals that a leucine repeat structure is required for binding to c-Jun, in a manner consistent with the proposed function of the "leucine zipper." A novel domain adjacent to but distinct from the leucine repeat of c-Fos is required for DNA binding by c-Fos–c-Jun heterodimers. Thus experimental evidence is presented that leucine repeats can mediate complex formation between heterologous proteins. This increases understanding of the molecular mechanisms underlying the function of two proto-oncogene products.

The human proto-oncogene product c-Jun is a component of the AP-1 family of transcription factors that mediates the regulation of gene expression in response to extracellular signaling events. Dr. Tjian and his colleagues have carried out a biochemical study of the relationship between the structure and function of c-Jun and its viral counterpart, v-Jun. Most interestingly, comparison of c-Jun and v-Jun by *in vitro* transcription assays revealed that the viral oncoprotein has significantly greater transcriptional activity than c-Jun. Analysis of a collection of Jun mutants expressed in bacteria

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indicates that the difference in transcriptional activity of c-Jun versus v-Jun may be due to the presence of a regulatory domain located at the amino-terminal region of c-Jun. These results suggest that during retroviral transduction a constitutively active Jun protein has been generated by deleting a negatively acting domain. This putative repressor domain may also play a role in the signal-dependent induction of c-Jun activity. Deletions and point mutants of Jun identify an activation domain rich in acidic and proline residues toward the carboxyl-terminal end of the molecule in a region adjacent to the DNA-binding domain.

III. Identification of a Novel Type of Proline-rich Activator.

Human CTF/nuclear factor I (NF-I) consists of a family of CCAAT box binding proteins that activate both transcription and DNA replication. Analysis of cDNA mutants expressed in *Escherichia coli* and *Drosophila* cells reveals that the amino-terminal portion of CTF-1 is sufficient for site-specific DNA recognition, protein dimerization, and adenovirus replication. In contrast, transcriptional activation requires an additional carboxyl-terminal domain. Furthermore, this transcription domain efficiently activates a heterologous promoter, such as SV40, when fused to the DNA-binding domain of Sp1. The CTF carboxyl-terminal region consists of an unusual type of transcriptional activation domain containing ~25% proline residues. Dr. Tjian and his colleagues propose that this proline-rich domain represents a novel class of activators that is distinct from activators containing either acidic or glutamine-rich activation motifs. This indicates that transcriptional activation is likely to be mediated by several different mechanisms. In addition, these results suggest that the interactions, and consequently the mechanisms, governing transcriptional activation by CTF are distinct from those mediating DNA replication.

IV. Mechanism for Negative Regulation in the *Drosophila* Embryo.

The *even-skipped* (*eve*) gene is a homeodomain-encoding gene that is a genetically defined repressor of *Ultrabithorax* (*Ubx*), *fushi tarazu* (*ftz*), and *wingless* (*wg*). Recently, Dr. Tjian and his colleagues have shown that purified *eve* protein represses transcription *in vitro* at the *Ubx* promoter, in a DNA-binding site-dependent manner. The *eve* protein represses transcription when bound either upstream or downstream of the RNA start site or when DNA-binding sites are in either orientation. Furthermore, *eve* represses expression from the *Ubx* promoter in *Drosophila* tissue culture cells, again in a binding site-dependent manner. Deletion of *eve* DNA-binding sites does not alter transcription in the absence of *eve*; thus repression is not likely to be the result of *eve* competitively inhibiting an activator protein from binding to the same DNA element. Instead, Dr. Tjian proposes that *eve* protein is probably interfering with the function of proteins bound at other locations in the promoter. The biochemical demonstration that a *Drosophila* homeodomain protein can directly regulate RNA synthesis strengthens the view that these regulators act as transcription factors to control development.

V. Analysis of a Developmentally Regulated *Drosophila* Transcription Factor Involved in Neuronal Gene Expression.

In an effort to characterize sequence-specific transcription factors that regulate gene expression during *Drosophila* development, Dr. Tjian and his colleagues have identified and purified a novel DNA-binding activity [neurogenic element binding transcription factor 1 (NTF-1)]. The purified protein consists of several polypeptides that bind selectively to a functionally important cis-control element of the *Ubx* promoter and to the neurogenic elements of both the dopa carboxylase (Ddc) and *ftz* promoter/enhancer regions. Purified NTF-1 activates transcription *in vitro* in a binding site–dependent manner through upstream sequences of the *Ubx* promoter. A cDNA clone encoding the open reading frame of NTF-1 was isolated, and the deduced primary amino acid sequence of NTF-1 includes a glutamine-rich region reminiscent of the transcriptional activation domains found in Sp1 but no recognizable DNA-binding domain. NTF-1 expression is temporally regulated during embryonic development. In addition, *in situ* hybridization experiments revealed that NTF-1 is transcribed in a spatially restricted pattern in the embryo, with the highest level of expression observed in the epidermis and a subset of cells in the central nervous system.

Expression of the NTF-1 cDNA in mammalian cells yields a protein that displays DNA-binding and transcriptional activities indistinguishable from those of the collection of proteins isolated from

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Drosophila embryos. These findings suggest that NTF-1 is a member of a family of developmentally regulated transcription factors that may be involved in directing the expression of genes such as *Ubx*, Ddc, and *ftz* in neuronal cells.

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

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GENETIC DEFECTS IN THE METABOLIC PATHWAYS INTERCONNECTING THE UREA AND TRICARBOXYLIC ACID CYCLES

DAVID VALLE, M.D., *Investigator*

Dr. Valle is interested in human biochemical genetics, with the objective of understanding the molecular basis, heterogeneity, pathophysiology, and treatment of a variety of inborn errors. During the past year he has continued his studies of the molecular biology, structure-function relationships, and inherited defects of ornithine-δ-aminotransferase. Deficiency of this mitochondrial matrix enzyme causes gyrate atrophy of the choroid and retina, a rare, autosomal recessive, blinding condition associated with accumulation of ornithine in all body fluids. He has also analyzed the phenylalanine hydroxylase gene in black Americans with phenylketonuria, with the goal of determining the molecular basis and origin of the phenylalanine hydroxylase mutant allele(s) in this population. Finally, Dr. Valle has collaborated in a study of the molecular basis of pseudohypoparathyroidism, a dominantly inherited inborn error in calcium homeostasis.

I. Gyrate Atrophy of the Choroid and Retina.

A. *Molecular and cell biology of ornithine-δ-aminotransferase (OAT).* To understand better the regulation of OAT transcription, John Engelhardt, a student in Dr. Valle's laboratory, has constructed a set of hybrid OAT promoter chloramphenicol acetyltransferase (CAT) constructs containing variable regions of the immediate 5′-flanking region of the OAT gene. Transfection of these hybrid constructs into a variety of mammalian cells has shown that the 5′-flanking region to −134 (where +1 is the transcriptional start site) is sufficient for maximal promoter activity. Furthermore, constructs containing this same region transfected into human retinoblastoma cells (Y79) but not human liver or kidney lines exhibit a 3–5 times increased expression in response to estrogen. The same construct exhibits cAMP-dependent expression (3–5 times) in human and rodent liver cell lines and in human kidney cells. These observations are consistent with the prediction from sequence analysis of possible estrogen and/or cAMP cis-acting elements in a 32 bp region overlapping the transcriptional start site (−25 to +7) that exhibits incomplete dyad symmetry and has homology with both estrogen and cAMP responsive elements. DNase I footprint analysis of this same region with nuclear extracts from various cell lines shows clear footprints over three Sp1-binding sites and to the CAAT box at −73. However, a clear footprint over the suspected estrogen and/or cAMP responsive elements has not been detected. This may indicate that the candidate sequence is not a region of protein binding or, more likely, that the binding is more complex (perhaps involving interaction of several proteins) and the correct conditions have not yet been produced. Additional footprinting, gel-shift, and mutagenesis experiments are in progress to clarify this point.

Dr. Valle and his colleagues have also found that alternative splicing of OAT mRNA occurs: most mature transcripts lack exon 2, an 87 bp exon in the 5′-untranslated region of the OAT mRNA, even though it is flanked by acceptable splice consensus sequences. Dr. Valle and his colleagues have now found that <5% of the OAT mRNA in a variety of human cell lines and tissues contains exon 2, and this proportion does not change when OAT expression is stimulated by cAMP in HepG2 cells. By contrast, >50% of OAT transcripts in mRNA isolated from a variety of monkey cell lines and tissues contain exon 2. Thus, as has been shown for the metabolically related enzyme argininosuccinate synthetase, there is a species-dependent, quantitative difference in the splicing pattern of OAT mRNA. Whether this is related to cis-acting, sequence differences in the relevant introns between monkey and human OAT or due to trans-acting differences in the splicing apparatus is being investigated.

B. *Analysis of the mutations causing gyrate atrophy (GA) of the choroid and retina.* Dr. Valle and his colleagues have characterized the OAT mRNA and OAT antigen phenotype in fibroblast samples from 72 GA pedigrees (27 Finnish, 45 non-Finnish), 92% of which express a normal or only moderately reduced amounts of normally sized OAT mRNA. Of the $mRNA^+$ probands, 67% are CRM^-; all of the $mRNA^-$ lines are also CRM^-. Using a combination of RNase A protection assays and polymerase chain reaction (PCR) amplification and sequencing of OAT exons in genomic DNA or fibroblast OAT cDNA, Dr. Valle and his co-workers have delineated 15 different mutant alleles that account for 75% or 52% of the 144 possible abnormal alleles from probands in the 72 pedigrees. Two alleles recently identified include one with two missense mutations in close proximity (20 codons apart) and a second with a

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142 bp inserted sequence precisely between exons 3 and 4 that has homology to Alu. This appears to be due to a mutation creating a new splice site within an Alu sequence in intron 3. Unexpected allelic heterogeneity has been shown in the 27 Finnish pedigrees, where at least three and possibly five different mutant alleles are found. By mapping and analyzing several polymorphic markers within the gene, Dr. Valle and his colleagues have also shown that one of the mutations discovered in Finns has occurred twice: once in ancestors of two Finnish families and again in ancestors of an American pedigree with no known Finnish ancestry. The functional consequences of these mutations in both mammalian and yeast cells and additional aspects of the distribution of the alleles in various populations are being investigated.

C. *Analysis of the OAT-hybridizing sequence on the X chromosome.* In collaboration with Dr. H. Lehrach, Dr. Valle has obtained a series of 22 X-linked cosmid clones that contain the X-linked OAT-hybridizing sequence. These are to be analyzed to produce an overlapping restriction map of the clones and to search for possible related functional genes in this region.

D. *Models for GA of the choroid and retina.* Lawrence Brody, a student in Dr. Valle's laboratory, has produced a transgenic animal using human OAT constructs that exhibit dominant negative expression characteristics in cultured cells. The transgenic animals will be analyzed to determine if expressing these mutant human OAT genes inhibits the activity of the endogenous murine OAT.

In collaboration with investigators at the University of Pennsylvania School of Veterinary Medicine, Dr. Valle and his colleagues have also identified a second domestic cat with GA; work is in progress to attempt to develop a colony of these cats for experiments related to pathophysiology and treatment of GA.

II. Phenylalanine Hydroxylase Mutations in Black American Phenylketonuric Patients.

To address the question of the nature and origin of the phenylalanine hydroxylase mutations causing phenylketonuria in black Americans, Dr. Valle and his associates have determined the restriction endonuclease haplotypes for this gene in 16 black phenylketonuric patients and compared them with a large series of black and Caucasian controls. Four new haplotypes have been observed, and a unique *Msp* site has been detected in five phenylalanine hydroxylase genes from four black patients. This site is a polymorphism in extreme linkage dysequilibrium with a previously undescribed missense mutation in exon 7 that appears to be the disease-producing mutation.

III. Molecular Basis of Pseudohypoparathyroidism.

Pseudohypoparathyroidism (pHP), an autosomal dominant disorder in calcium homeostasis, is characterized by short stature, mild intellectual impairment, and renal unresponsiveness to parathyroid hormone. Dr. Michael Levine obtained biochemical and mRNA evidence implicating the $G_s\alpha$ subunit of the G protein as the site of the primary defect in this disorder. Together, Drs. Valle and Levine and their colleagues studied an affected mother and son and showed that both are heterozygous for an initiator codon mutation in $G_s\alpha$, thereby confirming the hypothesis that mutations at this locus can produce the pHP phenotype.

Dr. Valle is also Professor of Pediatrics and of Molecular Biology and Genetics at The Johns Hopkins University School of Medicine.

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MOLECULAR BASIS OF DISEASE

CORNELIS VAN DOP, M.D., PH.D., *Assistant Investigator*

Dr. Van Dop's laboratory is concerned with molecular processes that alter cellular responsiveness to hormones. Particular emphasis is given to guanine nucleotide-binding coupling proteins and their alterations in disease. A second area of study relates to molecular lesions of adrenal steroid 21-hydroxylase that produce congenital adrenal hyperplasia.

I. Guanine Nucleotide-binding Proteins (G Proteins).

A variety of extracellular signals alter metabolism of mammalian cells by interacting with specific extracellular receptor proteins that then activate a guanine nucleotide-binding coupling protein (G protein). The activated G protein then modulates an intracellular second messenger system or an ion channel. The G proteins, a family of homologous proteins, share a heterotrimeric structure in which the α-subunit confers functional specificity and their common βγ-subunit ties together functions of different G proteins. Dr. Van Dop's laboratory has been studying several human diseases that result in part from altered G protein function. These diseases include pseudohypoparathyroidism type Ia and heart failure.

A. *Molecular lesions of G proteins in patients with pseudohypoparathyroidism type Ia.* Patients with pseudohypoparathyroidism type Ia and Albright's hereditary osteodystrophy commonly have a genetic deficiency of the α-subunit of the G protein that stimulates adenylate cyclase (αG_s). In most affected families, αG_s deficiency and Albright's hereditary osteodystrophy are transmitted together in a dominant inheritance pattern, although several families with autosomal recessive inheritance, and therefore probably a different causative genetic defect, have been described. Studies continue to determine the molecular defects in the genes encoding the αG_s that are causative for this disease.

B. *Retinal transducin.* The cDNA encoding the α-subunit of human retinal transducin has been cloned. This rod transducin cDNA shares 88% nucleotide sequence homology with the previously cloned bovine rod transducin. The amino acid sequence is also highly homologous between these two species, with identical sequences around the ADP ribosylation sites for the bacterial toxins from *Bordetella pertussis* and *Vibrio cholerae*.

C. *G protein alterations in heart failure.* Idiopathic heart failure, a pathophysiologic abnormality of heart muscle, diminishes the pumping function of the heart, resulting in inadequate delivery of blood to tissues and/or circulatory congestion. In heart failure the heart muscle manifests reduced responsiveness to the chronotropic and inotropic effects of sympathetic nervous system stimulation. Because cAMP modulates cardiac contractility and β-adrenergic receptors are the primary regulators of adenylyl cyclase in the heart, components of hormone-regulated adenylyl cyclase in human hearts with idiopathic heart failure were investigated. In collaborative studies with Dr. Arthur Feldman (The Johns Hopkins University), Dr. Van Dop and his colleagues examined the molecular basis for diminished cardiac responsiveness to β-adrenergic agonists in hearts from patients with idiopathic heart failure who had undergone cardiac transplantation and in hearts from Syrian hamsters that develop a heritable dilated cardiomyopathy.

Increased activity of the inhibitory guanine nucleotide-binding regulatory protein (G_i) in hearts from patients with idiopathic dilated cardiomyopathy had previously been demonstrated. Steady-state levels of the mRNA encoding the α-subunits of several G proteins were quantified, using Northern and dot-blot analysis. Steady-state levels of mRNAs encoding α-subunits of both G_i-3 and G_s were significantly increased in the failing hearts compared with nonfailing, control hearts. Among the three different αG_i subtypes, mRNA encoding αG_i-3 was most abundant, that encoding αG_i-2 was barely detectable, and that encoding αG_i-1 was not detectable. Coupled with the previous functional studies of G proteins in these same failing hearts, these results suggest that G protein function in the heart is modulated at several levels.

The cardiomyopathic Syrian hamster has been widely studied as a model for congestive heart failure in humans. These hamsters inherit as an autosomal recessive trait the propensity to develop congestive heart failure. Regulation of cardiac adenylyl cyclase in these animals was investigated before and after they developed heart failure. Pharmacologic stimulation of adenylyl cyclase in cardiac membranes by site-specific agents demonstrated

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defective coupling of G_s to adenylyl cyclase. This diminution of G_s bioactivity was not associated with reduced immunologic levels of αG_s to adenylyl cyclase. The defect in G_s bioactivity was limited to cardiac and skeletal muscle, occurred only in animals homozygous for the dystrophic trait, and was demonstrable before any cardiac abnormalities were evident on light microscopic examination of the hearts. The molecular basis for this functional alteration of G_s remains under investigation.

Finally, acute ethanol ingestion diminishes cardiac contractility; therefore Dr. Van Dop and his colleagues investigated the direct effects of ethanol on adenylyl cyclase activation in isolated cardiac membranes from normal hearts. Ethanol had no effects on basal activity or on manganous ion-mediated activation of adenylyl cyclase. However, activation of adenylyl cyclase by the nonhydrolyzable guanine nucleotide analogue guanylyl imidodiphosphate and by fluoride ion was enhanced by ethanol. Similarly, ethanol also increased activity of adenylyl cyclase in membranes that had been pretreated with isoproterenol and guanylyl imidodiphosphate. Ethanol thus appears to enhance acutely the coupling between activated G_s and the catalytic subunit of adenylyl cyclase. Further investigations of the mechanisms by which ethanol diminishes excitation-contraction coupling in the heart continue.

II. Molecular Lesions of Steroid 21-Hydroxylase in Salt-losing Congenital Adrenal Hyperplasia.

Genetic deficiency of the enzyme 21-hydroxylase is the most frequent cause of salt-losing congenital adrenal hyperplasia (CAH), an autosomal recessive disorder of adrenal steroidogenesis. The CAH locus, located within the major histocompatibility complex between HLA-B and HLA-D on chromosome 6p, consists of a tandem duplicated pair of genes encoding steroid 21-hydroxylase (CYP21) and the two fourth components of complement (C4A and C4B). One 21-hydroxylase gene (*CYP21B*) is expressed while the second gene (*CYP21A*) is a highly homologous pseudogene containing several deleterious mutations that prevent expression of active enzyme. Salt-losing CAH, the most severe form of CAH, frequently occurs with deletion of the *CYP21B* gene, which apparently results following unequal crossing over between the active gene and the pseudogene. In collaboration with Drs. Claude Migeon and Patricia Donohoue (The Johns Hopkins University), Dr. Van Dop has mapped the crossover sites in chimeric, recombinant *CYP21* genes from six patients with salt-losing CAH. Nucleotide sequences unique to the *CYP21A* pseudogene or to the active *CYP21B* gene were mapped, using gene-specific restriction sites and oligonucleotide hybridizations. Each chimeric *CYP21* gene in the *CYP21*-deletion–linked haplotypes contained sequences near the 5′ end that were characteristic of *CYP21A* and only a single transition of sequences of *CYP21A* to sequences of *CYP21B* to the 3′ end. These nucleotide sequence transitions all occurred within one of two discrete regions. All eight chimeric *CYP21* genes coupled with HLA-Bw47 in five unrelated patients had the *CYP21A-CYP21B* sequence transition within the same gene region (+1,375 to +1,993). One of the three other *CYP21B*-deletion haplotypes had a sequence transition within this same region, while in two other haplotypes the transition occurred between base pairs +470 and +999. Similar analysis of a haplotype with a gene conversion of the *CYP21B* gene to *CYP21A* suggests that sequential, multiple crossings over or a single gene conversion affecting a long segment of the *CYP21B* gene produces the gene conversion genotypes that also occur in patients with salt-losing CAH.

Dr. Van Dop was Assistant Professor of Pediatrics at Harvard Medical School and Director of the Endocrinology and Clinical Research Center Core Laboratories at Children's Hospital in Boston when these studies were conducted. He is now Associate Professor of Pediatrics at the University of California School of Medicine at Los Angeles.

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GENETIC LINKAGE MAPPING IN THE SEARCH FOR DISEASE GENES

RAYMOND L. WHITE, PH.D., Investigator

I. Human Genetic Linkage Map.

A. *Primary maps.* Efforts over the past several years have culminated in a nearly complete primary genetic linkage map of the human genome. In a primary map, genetic markers are spaced in such a way that any gene that in mutant form causes an inherited disease can be localized to a specific segment of a chromosome by the cosegregation of the mutant allele with a polymorphism of a nearby mapped marker, if sufficient DNA samples are available from families segregating the defective allele. Primary maps of chromosomes are constructed from 77 genetic linkage data obtained from a panel of 60 three-generation reference families, most of whom have been ascertained in Utah. During the past year, Dr. White's laboratory has published primary maps of chromosomes 1, 9, 10, 15, 18, and 19; when other maps appear that are currently in press or in preparation, this laboratory will have contributed primary mapping data for all but the two smallest chromosomes, 21 and Y.

B. *High-resolution maps.* Once primary maps are established, the next step in linkage analysis (as mandated by the Genome Project) is to develop high-resolution maps of markers ~1 centimorgan apart, the limit of resolution of linkage studies. High-resolution maps make it possible to pinpoint with greater accuracy an unknown gene that has initially been localized to a chromosomal region by primary mapping and to narrow the target sufficiently that other techniques can be used to isolate and characterize the gene. High-resolution maps will also serve as the basis for ordering sets of overlapping cosmids into physical maps of the chromosomes. This laboratory has been concentrating on chromosomes 16 and 17 for these high-resolution mapping studies.

This year the laboratory has automated enzyme digestion and gel loading. Libraries of clones from flow-sorted chromosome 16 (provided by the Los Alamos National Laboratory) are being searched, with the goal of identifying 100 new polymorphic marker systems for this chromosome.

Thirty new DNA markers based on loci containing a variable number of tandem repeats (VNTRs) have been developed for chromosome 17, by hybridization of a synthetic oligonucleotide sequence (GGNNGTGGG), under conditions of low stringency, to cloned chromosome 17 sequences derived from somatic cell hybrids. These and an additional 35 marker loci that show site polymorphism with two or more enzymes and have an average heterozygosity >70% are being genotyped in the 60 reference families for eventual ordering into a high-resolution map of this chromosome.

II. Disease Linkages.

The large number of polymorphic markers developed in this laboratory, in particular the several hundred highly polymorphic VNTR markers now available for the genomic map, make detection of new disease gene locations, by this and other laboratories worldwide, an ongoing and accelerating process. This year, genetic studies under the direction of Dr. Mark Leppert detected linkage between markers on chromosome 20 and a gene responsible for a syndrome of benign familial neonatal convulsions; Dr. Leppert's group also showed that the genetic lesion in a large kindred that shows a complex phenotype, including colon cancer in some individuals, is at the same locus as the gene on chromosome 5 that is responsible for adenomatous polyposis coli (APC) in other families. In addition, DNA markers developed here have been used for high-resolution mapping in the vicinity of genes responsible for multiple endocrine neoplasia types 1 and 2A. VNTRs have also contributed to studies in collaborating laboratories that have detected loss of heterozygosity in tumor cells as a way to pinpoint the molecular changes leading to cancer.

III. Adenomatous Polyposis Coli.

The first of two genes actively being sought in this laboratory is the locus on chromosome 5 that harbors a mutation leading to familial APC. Dr. Yusuke Nakamura's group, after constructing a high-resolution linkage map of markers in the region of the APC gene, isolated 50 cosmid clones within a 5 Mb region containing the gene and ordered them into a physical map. This group is attempting to identify mutations within the region in 40 sporadic cases of adenomatous polyposis, by looking for new sizes of fragments on Southern blots and pulsed-field gels. They have also isolated cDNA clones from the region that express normal activity in colon tissue, to identify genes that might be candidates for harboring the APC mutation.

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iV. von Recklinghausen Neurofibromatosis.

Dr. Peter O'Connell's group is seeking to identify the gene responsible for peripheral neurofibromatosis (NF1). They have constructed a high-resolution genetic linkage map in the vicinity of the NF1 locus near the centromere of chromosome 17 and a physical map of cosmids in the region, as vital tools for the search. An important breakthrough was provided by the observation of translocations involving the appropriate region of chromosome 17 in the cells of two separate NF1 patients. Both translocation breakpoints were found to reside on one 600 kb fragment of DNA, narrowing the region containing the gene to a segment that is amenable to molecular investigation. A gene identified as the human homologue (*EVI2*) of a murine proto-oncogene, *evi-2*, was cloned and mapped between the translocation breakpoints; the pattern of expression of *EVI2* in human tissues is consistent with a potential role for this gene in NF1.

Dr. White is also Professor of Human Genetics and 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 focus of research in Dr. Williams's laboratory has been the use of gene transfer to study hematopoiesis and hematopoietic stem cell behavior. One long-term goal is the application of gene transfer technology for the treatment of certain human genetic diseases involving the bone marrow. A clear understanding of hematopoietic stem cell biology may play a key role in successful application of somatic gene therapy for treatment of such diseases.

I. Gene Transfer into Hematopoietic Stem Cells.

Dr. Williams's laboratory and other researchers have studied the use of recombinant retroviral vectors to transfer the adenosine deaminase (ADA) cDNA into murine hematopoietic stem cells. Deficiency of ADA is associated with severe combined immunodeficiency disease (SCID), a rare and fatal genetic disease of children. ADA deficiency is one disease for which somatic gene therapy offers a therapeutic option in the future and thus serves as a model for the development of gene transfer methods.

Dr. Williams, in collaboration with Dr. Stuart H. Orkin (HHMI, Harvard Medical School), has previously used recombinant retroviral vectors to transfer and express human ADA in murine hematopoietic stem cells. However, due to low efficiency of gene transfer, the stability of expression of human ADA in mice receiving bone marrow transplants with the modified stem cells was not examined. Recently, Drs. Bing Lim and Jane Apperley in Dr. Williams's laboratory have improved the efficiency of gene transfer into hematopoietic stem cells. Subsequently, Dr. Lim's work has shown that ~30–40% of mice transplanted with hematopoietic stem cells containing the human ADA cDNA express human ADA protein after full reconstitution following bone marrow transplantation. The level of expression varies from 10% to 100% of endogenous murine ADA enzyme expression and appears stable over time in these animals.

Although these results are encouraging, improvement in the efficiency of gene transfer into long-lived reconstituting hematopoietic stem cells is needed. Such improvements could theoretically lead to long-term expression of the transferred ADA cDNA in all mice after reconstitution and is critical for development of gene transfer for use in larger species, including primates and humans. One approach to improving gene transfer into hematopoietic stem cells is to provide the optimal conditions for such stem cells *in vitro*, during the gene transfer procedure.

II. Hematopoietic Stem Cell Interaction with the Hematopoietic Microenvironment.

Hematopoietic stem cell survival and proliferation, both *in vitro* and *in vivo*, is dependent on direct interaction of the stem cell with cells making up a complex environment in the bone marrow. Dr. Williams's laboratory has previously shown that recombinant retroviral vectors containing certain oncogenes are useful for the immortalization and subsequent cloning of stromal cells from this microenvironment of murine bone marrow. Several immortalized cell lines effectively replace the complex microenvironment in the support of reconstituting hematopoietic stem cells *in vitro*. Dr. Carmella Stephens has used these cell lines to begin to elucidate at the molecular and biochemical level the protein(s) involved in hematopoietic stem cell–stromal cell interactions. Further characterization of this interaction may lead to the elucidation of growth factors associated with the hematopoietic microenvironment and responsible for stem cell survival *in vitro*.

An additional application of immortalized stromal cell lines that support hematopoiesis *in vitro* relates to gene transfer experiments. Gene transfer into hematopoietic stem cells requires extensive *in vitro* manipulation and, at times, drug selection. Stromal cell lines will provide an optimal microenvironment for culturing stem cells for prolonged periods during *in vitro* manipulations. Dr. Barry Luskey is exploring the use of an immortalized primate stromal cell line produced in Dr. Williams's laboratory for selection of primate and human hematopoietic stem cells after retroviral-mediated gene transfer.

The interaction of hematopoietic stem cells and the hematopoietic microenvironment is likely to be important during embryonic development. During the initial stage of embryonic hematopoiesis, pluripotent hematopoietic stem cells in the fetal yolk sac are restricted to erythroid differentiation. This restricted differentiation is being investigated, using transgenic mice generated by embryonic stem cells in which specific myeloid growth factors are

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overexpressed in the fetal yolk sac. The hematopoietic microenvironment in the fetal yolk sac is also being studied, using cell lines generated from mesoderm tissue in which hematopoiesis is known to originate.

III. Gene Transfer and Targeting in Embryonic Stem Cells.

Embryonic stem (ES) cells are totipotent cells derived from day 3.5 murine blastocysts. Such cells can be manipulated *in vitro* and reintroduced into embryos by microinjection. The introduced cells contribute to somatic and germline chimerism and thus can be used to generate transgenic mice. Homologous recombination methods can be used to target specific sequences in the ES cells for disruption. Dr. Williams's laboratory is using ES cells for generation of transgenic mice to study fetal hematopoiesis. In addition, ES cells are being used to study the effect of lineage-specific expression of the growth-regulating proto-oncogene, c-*Myc*. A long-term goal is the generation of murine models of human diseases, using gene targeting methods in ES cells.

Dr. Williams is also Assistant Professor of Pediatrics, Harvard Medical School, and Associate in Hematology-Oncology, Children's Hospital, Boston, and Clinical Associate at the Dana-Farber Cancer Institute.

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MOLECULAR GENETICS OF HUMAN DISORDERS

SAVIO L. C. WOO, PH.D., *Investigator*

Dr. Woo's laboratory is interested in phenylketonuria (PKU), a genetic disorder that predisposes affected children to develop severe mental retardation. The disorder is secondary to a genetic deficiency of hepatic phenylalanine hydroxylase (PAH). It is transmitted as an autosomal recessive trait and has a prevalence of ~1 in 10,000 Caucasians.

I. Molecular Basis and Population Dynamics of PKU.

A. *Molecular lesions of prevalent mutant PAH alleles in Caucasians.* The PAH gene represents a highly polymorphic locus in the human genome, with more than 50 established restriction fragment length polymorphism (RFLP) haplotypes. In PKU patients throughout Europe, 75% of mutant PAH alleles are associated with haplotypes 1–4. The molecular lesions associated with mutant haplotypes 2 and 3 were characterized previously and shown to be in linkage disequilibrium with their respective haplotypes throughout the European continent. The results suggested a "founder effect" for the spread of PKU among Caucasians. More recently, the PAH locus of a compound heterozygote bearing mutant haplotype 1 and 4 alleles was analyzed by polymerase chain reaction (PCR) amplification of individual exons, followed by sequencing analysis. Two missense mutations were observed, and both were Arg to Gln substitutions. The mutation involving residue 158 is associated with mutant haplotype 4, while that in residue 261 is associated with haplotype 1. The linkage disequilibrium is again maintained throughout the European continent, supporting the concept of founder effect as the cause of spread of PKU in Caucasians. In this case, however, not all mutant haplotype 1 and 4 alleles are represented by these two mutations. The observation is not surprising, because haplotypes 1 and 4 are prevalent among normal alleles and could sustain multiple mutations during human evolution.

B. *Recurrent mutations in the PAH locus.* A Glu^{280} to Lys^{280} mutation has previously been observed in PKU patients from northern Africa; this mutation is in linkage disequilibrium with haplotype 38. The same mutation has recently been observed in two different families from northern Europe. One such mutant allele is associated with haplotype 1; the other is associated with haplotype 4. Because the three haplotypes are different in their individual RFLP distributions, the results suggest that recurrent mutations at the same site of the PAH gene might have occurred on different background chromosomes in different regions of the world. Examination of the nature of the mutation revealed a transversion event involving a CpG dimer, which is known to be a methylation site and more susceptible to mutations.

C. *Molecular genetics of PKU in Orientals.* Although PKU was originally thought to be a disorder restricted to Caucasians, recent implementation of a PKU-screening program in China showed that the disorder is also prevalent among Orientals (1 in 16,000). In that population, however, the majority of normal as well as mutant PAH alleles are associated with a single haplotype, haplotype 4. Consequently, RFLP haplotyping in that population is not as informative as it is in Caucasians. Molecular analysis of the mutant alleles by PCR amplification and direct DNA sequencing has revealed three distinct missense mutations: Arg^{111} to Ter^{111}, Tyr^{204} to Cys^{204}, and Arg^{413} to Pro^{413}. These three mutations are in linkage disequilibrium with mutant haplotype 4 and together represent ~30% of all PKU chromosomes in the Oriental population. The results provide conclusive evidence that multiple mutations occurred on the same haplotype background in the PAH locus and that there are prevalent mutant alleles in the Oriental population. Thus carrier detection may be possible in that population as well.

D. *PKU mutations occurred after racial divergence.* Oligonucleotides corresponding to the prevalent mutant alleles in the Caucasian and Oriental populations were used to analyze PKU patients from both continents. These mutant alleles are non-overlapping, providing conclusive evidence that the majority of PKU mutations must have occurred on the PAH locus in humans after racial divergence.

II. Somatic Gene Therapy of PKU.

The construction of a recombinant retrovirus bearing the human PAH cDNA under the transcription regulation of a liver-specific promoter has been reported previously. The recombinant retrovirus was capable of infecting primary mouse hepato-

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cytes in culture, and human PAH mRNA accumulated in the infected cells to a level comparable with that in normal human liver. The next area to be investigated is the reintroduction of such hepatocytes into living animals to determine their survivability, as well as functionality, *in vivo*. To investigate the efficacy of hepatocyte transplants and to avoid complications of immunological rejection of engrafted cells, Dr. Woo and his colleagues used a transgenic mouse model expressing high levels of human α_1-antitrypsin. This human protein is synthesized in the transgenic liver and secreted into blood, where it can be detected by a radioimmunoassay utilizing an antibody preparation that is specific for the human protein. Furthermore, the transgenic mouse was created in C57 black mice, and hepatocytes from these animals can be transplanted into nontransgenic C57 black mice, so that there will be no immunological rejections *in vivo*.

A. *Transplantation of hepatocytes in neovessels.* A variety of inert carriers have recently been reported in the literature that may serve as support for hepatocyte transplants. Cytodex 3 is a collagen bead onto which primary hepatocytes can be attached and injected into the peritoneal cavity. Alternatively, hepatocytes can be seated into a collagen/gelatin matrix that has previously been coated with an angiogenesis factor, and the gel foam can then be implanted onto the liver. Impressive vascularization into the neovessel occurs within days of transplant. With both methods, human α_1-antitrypsin can be readily detected in plasma of the transplanted mice. Unfortunately the level of the human protein in mouse plasma underwent a steady decline with time and became undetectable after several weeks. Retrieval of the neovessel and staining for glucose-6-phosphatase demonstrated that there were few hepatocytes left at that stage. Thus the neovessel does not appear to be a promising means of reintroducing primary hepatocytes into living animals.

B. *Transplantation of hepatocytes through the portal vein.* Primary transgenic hepatocytes were injected directly into the portal vein of congenic mice, and the production of human α_1-antitrypsin in plasma was observed within a day. The level continues to increase and reaches a plateau after about a week. This level is maintained after three months of transplantation, suggesting the transplanted hepatocytes must have established themselves *in vivo*. Furthermore, the level of human α_1-antitrypsin accumulation in mouse plasma suggests that 20% of the transplanted hepatocytes remain functional after transplantation. Although these results are preliminary, engraftment of virally infected hepatocytes may be explored as a means of somatic gene therapy for hepatic deficiencies such as PKU.

Dr. Woo is also Professor of Cell Biology and Molecular Genetics at Baylor College of Medicine.

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MOLECULAR STUDIES OF BEHAVIOR AND DEVELOPMENT IN *DROSOPHILA*

MICHAEL W. YOUNG, PH.D., *Investigator*

I. Neurogenesis.

A study of the genetic control of ectoderm development in *Drosophila* has been initiated through the recovery of neurogenic mutants. These overproduce neuroblasts at the expense of nonneural ectoderm. The best-characterized mutants in this series are associated with complete loss of function at the *Notch* locus. From its DNA sequence, *Notch* appears to encode a 288 kDa protein, the structure of which is dominated by a 36-fold tandem repetition of a cysteine-rich peptide related to epidermal growth factor (EGF). In *Notch*, no two of these repeats are identical, and genetic and molecular analyses of mutations affecting this domain of the protein indicate that differentiation of function exists among the different EGF-like segments. Genetic interactions between *Notch* alleles and between *Notch* and unlinked neurogenic loci can be modified by amino acid substitutions affecting specific EGF-like repeats of the Notch protein. All of this is consistent with the notion that Notch participates in ectodermal development through cell-cell interactions.

Dr. Young and his colleagues recently characterized the Notch protein itself biochemically and determined its distribution in developing *Drosophila*. Antibodies show that Notch is a stable, high-molecular-weight glycoprotein. This transmembrane protein, with the EGF-like elements exposed at the cell surface, is phosphorylated variably on serines of the cytoplasmic domain. Individual Notch polypeptide chains appear to be joined to each other by disulfide bonds, suggesting that homotypic interaction of these proteins is required for function. It is not known whether these interactions generally involve molecules on the same or neighboring cell surfaces.

Immunocytochemistry has shown that at the time of gastrulation, Notch is largely restricted to ectodermal cells defining the neurogenic region of the embryo. All of these cells have the potential to form neuroblasts, but only a fraction do so; the remainder become precursors of the epidermis in a lineage-independent fashion. Thus Notch appears to be associated with both epidermal and neural precursors. High levels of Notch expression eventually become restricted to neuroblasts, but only after these begin to delaminate from the embryonic ectoderm and come into close association with mesoderm. Mesodermal cells also produce Notch as neuroblast delamination proceeds, indicating a possible role in embryonic neurogenesis. During larval and pupal development, Notch becomes preferentially associated with nervous tissue and is expressed on stem cells, fully differentiated cells, and fasciculating nerve processes. Thus Notch seems to be present before and long after cell fate is determined. In the eye imaginal disk, Notch is switched on in the morphogenetic furrow. This is where cells that will compose ommatidia aggregate prior to establishing their specific developmental fates. As in embryonic neurogenesis, fate is determined by position, not lineage, and all cells make Notch. The protein continues to be associated with ommatidia after differentiation. Uniform expression on cells interacting to produce different developmental lineages from single primordia in the eye and embryonic ectoderm suggests that Notch alone may be insufficient to elaborate cell fates.

II. Biological Rhythms.

Periodic functions from heartbeat to 24 h (circadian) sleep/wake cycles are affected by mutations at the *per* locus. The 1,200–amino acid protein encoded by *per* shows some sequence similarity to vertebrate proteoglycans. Changes in a fly's rhythms can be produced by amino acid substitutions in the per protein, or by regulating *per* expression. In transgenic *Drosophila* a relationship between period length and abundance of the gene products is seen: high levels of expression lead to a short-period biological clock; low levels are linked to long-period behavior.

Cell-level phenotypes have been recognized for *per* mutants in the laboratory through studies of intercellular junctional communication in salivary glands. Low levels of *per* expression, and arrhythmic and long-period phenotypes, are correlated with poor intercellular junctional communication. Alternately, higher than wild-type levels of cell-to-cell communication are observed in mutant tissue from *Drosophila* having fast running biological clocks.

Cells making per protein in developing *Drosophila* have been identified by *in situ* hybridization to *per* transcripts and immunocytochemistry. Very low levels of protein are found in segmentally arranged clusters of cells composing part of the embryonic nervous system. Later in development, *per* RNA and

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protein are made in salivary glands, eyes, optic lobes, and the thoracic central nervous system. Unexpected locations also are found; high levels of protein are made in pupal and adult ovaries and testes, and the gene is expressed in some endocrine organs.

Some of the major sites of *per* RNA and protein synthesis can be correlated with genetically defined *per*-dependent functions, and this suggests that some phenotypes may be autonomously controlled by the identified cells. For example, the localization of *per* RNA and protein in the thoracic ganglia fits well with the observation that *per* is required in the thorax for production of ultradian rhythms connected with male courtship song. *per* RNA and protein are expressed in the adult optic lobes. Mutations affecting optic lobe development alter (and in some cases may eliminate) adult locomotor activity rhythms.

Some sites of expression suggest a wider range of phenotypic effects than previously reported. Synthesis in the ring gland complex is correlated with rhythmic secretion of an eclosion hormone, gating adult emergence from pupation. Possibly a *per*-dependent, autonomous pacemaker in this cell complex controls eclosion timing. Expression of *per* was found in gonads, and it has recently been shown that sperm release in some insects is controlled by a circadian pacemaker that functions *in vitro* in isolated testes.

A curious feature of the protein's expression is location in different subcellular compartments in different tissues. The protein is associated with nuclei in photoreceptor cells, optic lobes, and ring gland. It is cytoplasmic, and possibly on cell surfaces, in the embryonic nervous system, gonads, salivary glands, and certain cells of the central brain. The different localizations could point to involvement in more than one cellular process.

Dr. Young is also Professor of Genetics at The Rockefeller University.

PUBLICATIONS

Books and Chapters of Books

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Articles

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The Program in Immunology, one of the original disciplinary areas of the Institute, is represented at the University of Michigan, the University of Alabama at Birmingham, the Massachusetts Institute of Technology, Texas Southwestern Medical Center at Dallas, the National Jewish Center for Immunology and Respiratory Medicine at Denver, Duke University Medical Center, Baylor College of Medicine, the University of California at Los Angeles and at San Francisco, Yale University, Columbia University College of Physicians and Surgeons, Stanford University, the California Institute of Technology, the University of Washington, and Washington University in St. Louis. Among the topics being studied by investigators in this program are the development of the immune system and the mechanisms for generating immunological diversity, the structure and function of immunoglobulins and the T cell antigen receptors, regulation of the immune response, effector mechanisms in the immune response, and immunopathology.

Investigator Max D. Cooper, M.D. (University of Alabama at Birmingham) is interested in the normal and abnormal development of immunocompetent T and B cells in vertebrates. This laboratory has identified a third type of T cell in birds. Comparison of the antigen receptors on this recently discovered T cell with those recognized previously suggests the existence of T cell receptor subclasses, the genes for which are programmed for sequential rearrangement and expression. Also in birds, a thymus-independent line of lymphocytes has been identified that expresses certain T cell products but not the classical antigen receptors.

The research of Assistant Investigator Craig B. Thompson, M.D. (University of Michigan) and his colleagues is focused on characterizing the molecular events associated with the regulation of lymphoid development and proliferation. They report progress in determining how genetic diversity is created in the immunoglobulin genes of developing chicken B cells. This work provides insights into how genetic heterogeneity arises through the process of gene conversion. In addition, Dr. Thompson's laboratory continues to examine gene regulation during T cell proliferation. The laboratory has demonstrated a specific role for the regulation of mRNA stability in the regulation of lymphokine gene expression. Studies concerning the regulation of mRNA stability are under way.

Molecular genetic events involved in the ability of animals to produce antibodies have been examined by the laboratory of Investigator Frederick W. Alt, Ph.D. (Columbia University). Antibody genes are encoded in pieces in the germline; these genes must be assembled during development of antibody-producing cells. This laboratory has elucidated aspects of the mechanism and control of this gene assembly process that have provided fundamental insights into the way mice and humans generate a specific antibody repertoire and also the nature of genetic defects that impair this process. They have now applied new technologies to alter the genetic constitution of mice to create model systems and elucidate the molecular and physiological mechanisms regulating the development of antibody-producing cells and the generation of the immune response.

The research of Assistant Investigator Rudolf Grosschedl, Ph.D. (University of California at San Francisco) and his colleagues is focused on the mechanisms that allow immunoglobulin (Ig) μ and κ genes to be expressed only in one cell type and in a temporally ordered manner. By transfer of wild-type and mutated genes into the mouse germline, specific factor-binding sites in the enhancer and promoter were shown to be important for μ gene expression in lymphocytes. The temporal regulation of Ig gene expression was found to be mediated, at least in part, by the enhancer. This genetic element was also found to be a determinant for the stable propagation of the pattern of Ig gene expression from mother to daughter cells. Finally, cDNA clones representing novel lymphocyte-specific genes were isolated and partially characterized.

In addition to their role in the body's defense against invading organisms such as viruses and bacteria, T cells are also thought to mediate the development of certain autoimmune diseases and to participate in tissue or organ transplant rejection. Thus T cells must be able to recognize foreign invaders. However, these cells should not recognize and respond to the host animal itself, since such an event can lead to a number of autoimmune diseases. The laboratory of Investigator Philippa Marrack, Ph.D. (National Jewish Center for Immunology and Respiratory Medicine, Denver) is interested in the events that lead to the state where T cells can recognize foreign material, but not self. It has been found that events critical to this distinction usually occur in the thymus, the organ in which T cells develop. During T cell development

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those cells that recognize their own host tissues (autoreactive cells) die, and only those cells that are not autoreactive are allowed to become fully mature and escape into the circulation for distribution throughout the body, where they can contribute to disease resistance.

Associate Investigator Dennis Y. Loh, M.D. (Washington University) and his colleagues are also interested in thymus-derived lymphocytes. To understand how T cells develop under normal circumstances, genes important in T cell function have been cloned and re-introduced into transgenic mice. Results are providing insights into how T cells learn to discriminate self and nonself markers to maintain the balance of the body's immune system.

The laboratory of Associate Investigator Roger M. Perlmutter, M.D., Ph.D. (University of Washington) has defined a limited set of gene segments that contributes preferentially to the assembly of antibody-combining sites during fetal and neonatal life. Many of these early antibodies appear to be similar to antibodies found in patients with autoimmune diseases, indicating that pathologic autoantibodies may be part of the normal preimmune repertoire. Other studies have identified protein tyrosine kinases that behave as signal transduction elements in hematopoietic cells. Experiments in cell lines and in transgenic animals suggest that one of these ($p56^{lck}$) participates in signal transduction from the T cell antigen receptor complex.

T cells recognize the presence of foreign material, such as bacteria or viruses, in the body by means of receptors they bear on their surfaces. These receptors are made up of between five and eight components that can vary from one cell to another, so that one T cell can recognize poliovirus by means of its receptor, and another, measles. Usually a particular T cell must have the right combination of all five to eight components to recognize a particular invader; for example, an individual who has not previously been exposed to measles will have few reactive T cells and respond poorly to the virus when first infected. There are some exceptions, however, to this rule. So-called superantigens stimulate all T cells bearing a particular version of one of the components, regardless of the others. Among these exceptions are a collection of toxins produced by *Staphylococcus aureus* that cause food poisoning or toxic shock in humans. Because they are superantigens, these toxins are able to stimulate a large number of T cells. The toxin that causes toxic shock, for example, will react with about 10% of all T cells in a given individual, or approximately 100,000,000,000 cells. The laboratory of Investigator John W. Kappler, Ph.D. (National Jewish Center for Immunology and Respiratory Medicine, Denver) has predicted that it is the stimulated T cells that cause all or most of the symptoms arising from exposure to the staphylococcal toxins, rather than the toxins themselves.

Research in the laboratory of Investigator Charles A. Janeway, Jr., M.D. (Yale University) seeks to characterize the cellular interactions and transmembrane signaling events that lead to the selection of a self-tolerant and self MHC (major histocompatibility complex)-restricted T cell antigen receptor (TCR) repertoire. The demonstration in this laboratory that the TCR is oriented to the peptide-MHC ligand, that crosslinking and conformational change both contribute to T cell activation, and that self peptides are poorly represented in the thymic cortex has led Dr. Janeway to propose that either crosslinking or conformational change alone induced by TCR interactions with MHC molecules in the thymic cortex may generate a novel signal for positive selection of self MHC–recognizing T cells. These events are now being described in terms of signal transduction.

The long-term goal of Assistant Investigator Jeffrey M. Leiden, M.D., Ph.D. (University of Michigan) and his colleagues is to understand the molecular mechanisms that regulate gene expression during cellular differentiation and activation. Their studies of the regulation of the TCR α-chain gene have identified the genetic elements and protein factors that are responsible for specifically turning on the expression of this gene in human T cells. The laboratory has cloned one of the protein factors that regulate the expression of the TCR α-chain gene, as well as a number of other cAMP-responsive genes. Dr. Leiden is also interested in cardiac troponin C, the calcium-binding protein that regulates the force of contraction in the heart. The laboratory recently cloned the cardiac troponin C gene, analyzed its expression during muscle cell development, and produced monoclonal antibodies specific for this protein. These antibodies may be useful in the early diagnosis of patients with heart attacks.

The activation of T lymphocytes after antigen challenge is a central feature of the immune response. The studies of Associate Investigator Arthur Weiss, M.D., Ph.D. (University of California at San Francisco) and his colleagues are an attempt to understand how cell surface molecules, specifically the TCR, initiate T cell responses. Stimulation of

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the TCR induces several intracellular biochemical changes. The relationship between these changes and cellular responses has been revealed by experiments in which a foreign receptor has been introduced into a T cell line. The receptor for the neurotransmitter acetylcholine, normally expressed on nerve or muscle cells, was introduced into a T cell line. Stimulation of these T cells with acetylcholine induces well-characterized biochemical signals that are also induced by the TCR. These biochemical changes result in the activation of the T cell line, as measured by the secretion of lymphokines (hormones of the immune system) and expression of new growth factor receptors. Thus the signals that these two receptors both induce can lead to T cell activation. How the TCR induces these changes is not known. In an attempt to elucidate this process, a genetic approach has been used to isolate mutants of a T cell line that fail to respond to TCR stimulation. This laboratory also has identified two proteins that interact with the TCR after it has been stimulated. By characterizing the nature of these mutations and the molecules that interact with the TCR, insight into how T lymphocytes become activated to express their functional activities should emerge. This may lead to more rational approaches to regulating T cell activities in disease states.

The laboratory of Associate Investigator Mark M. Davis, Ph.D. (Stanford University) has developed a model of T cell recognition that could help to explain why the antigen receptors on T cells differ so dramatically from those on B cells. The model also suggests how antigen receptor genes could have evolved from more primitive cell surface molecules. The establishment of lines of mice carrying and expressing specific T cell receptor complexes promises to yield important information on receptor selection and control of expression during T cell development in the thymus.

Investigator Susumu Tonegawa, Ph.D. (Massachusetts Institute of Technology) and his colleagues have continued to study the nature and function of T cells bearing the second type of receptor, γδ TCR. They found that these T cells are localized in many epithelial tissues, including those in the reproductive and digestive organs. The TCR expressed in some of the epithelia are structurally undiversified, while those expressed in others are diverse. This laboratory also showed that a DNA element associated with the TCR γ gene plays a critical role in the cell lineage determination of αβ and γδ T cells. A definition of the nature of the molecules (ligands) recognized by γδ T cells is also sought.

Lymphocyte interactions during an immune response are necessary for the activation of antigen-specific lymphocytes. The resulting effector phase of an immune response is described as being either humoral or cell mediated. These effector mechanisms are well characterized, yet the precise mechanisms by which the response to a given antigen or infectious agent is directed into the humoral or cell-mediated mode are not known. What is clear, however, is that both types of immunity depend on the activation of CD4 T cells, which were originally shown to have multiple functions, including helping B cells proliferate and secrete antibody, mediating delayed-type hypersensitivity reactions, proliferating *in vitro* to exogenous antigens, and inducing CD8 cytolytic T cells. Thus it was questioned whether the same CD4 T cell could mediate all these functions, thus encompassing both humoral and cell-mediated responses, or whether the CD4 T cell population was functionally heterogeneous. CD4 T cell functional heterogeneity and the activation conditions that lead to this heterogeneity have been a central focus of the laboratory of Associate Investigator Kim Bottomly, Ph.D. (Yale University).

The major research interest of Assistant Investigator Dan R. Littman, M.D., Ph.D. (University of California at San Francisco) is the function of the T cell surface molecules CD4 and CD8. These molecules bind to different types of histocompatibility molecules and transmit signals required for the choice of developmental pathways during thymocyte development. Dr. Littman's laboratory is studying the molecular basis of the interaction of CD4 and CD8 with the histocompatibility molecules, with T cell receptors, and with intracellular protein kinases involved in transducing signals. The CD4 molecule also serves as the major receptor for the human immunodeficiency virus (HIV). Genetic and structural approaches are being used to study the basis of the interaction of CD4 with the HIV envelope protein. Genetic approaches are also being employed to identify other receptors involved in HIV entry. Information gained from these studies may allow design of derivatives of these receptors that can interfere with HIV infection.

The laboratory of Investigator Warner C. Greene, M.D., Ph.D. (Duke University) also studies the molecular and biochemical basis for the activation and growth of cells that form the body's immune defense. Recent attention has focused on various disease-causing viruses that interrupt this defense network, including human retroviruses that cause

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cancer (human T cell leukemia virus type I, HTLV-I) and AIDS (human immunodeficiency virus type 1, HIV-1) and the pathological interplay of these viruses with immune cells.

Assistant Investigator B. Matija Peterlin, M.D. (University of California at San Francisco) studies the copying of genes that are important for normal host defense mechanisms. Steps leading to the activation and amplification of spread of the virus that causes AIDS (HIV-1) also are investigated. Biochemical and genetic techniques are used to isolate, clone, characterize, and express genes that are important in these regulatory processes. These studies should lead to the identification of the genetic factor that is missing in the bare lymphocyte syndrome, a disease where children die at a young age of immunodeficiency. They also should increase our understanding of mechanisms involved in HIV latency and progression to active disease.

In the past year Assistant Investigator Dorothy E. Lewis, Ph.D. (Baylor College of Medicine) has focused on understanding the effects of HIV on $CD8^+$ cells in an *in vitro* model system. The cells are productively infected in this system, but it is unclear whether the cells are infected via a $CD4^+$ cell or by another mechanism. This laboratory also is examining plant phospholipids for their effect on HIV production *in vitro*. The compounds are effective in their interference and relatively nontoxic. Future experiments will explore the mechanisms responsible for this interference with HIV production. In addition, Dr. Lewis has developed a sensitive *in situ* hybridization technique. In her study of more than 50 HIV-infected individuals she reports that, in 10 of 25 AIDS patients, more than 30 out of 10,000 cells were transcriptionally active. Most importantly, increased numbers of transcriptionally active cells appear to be correlated with the patient's clinical condition: this would imply that viremia *per se* could account for the pathology of HIV infection.

The laboratory of Associate Investigator David D. Chaplin, M.D., Ph.D. (Washington University) is investigating the structure of the human major histocompatibility complex (MHC). Genes within the MHC participate in essentially all phases of the immune response. The new yeast artificial chromosome (YAC) DNA cloning technology is being used to determine the relationship of all the genes within the MHC. YAC clones that define more than half of the gene complex have been isolated, and these will permit a detailed analysis of the structure of the complex. Dr. Chaplin also studies the molecular and cellular biology of interleukin-1, an important immunomodulatory cytokine. His laboratory has produced new reagents that should permit biochemical analysis of this agent in a variety of immunologic systems.

The MHC class II molecules play a pivotal role in determining both normal and abnormal immune responses. The expression of class II genes is regulated by a series of genetic elements and a series of soluble proteins that bind to these elements. The cDNAs encoding two such proteins, YB-1 and YB-2, have been isolated, characterized, and sequenced by Investigator Benjamin D. Schwartz, M.D., Ph.D. (Washington University) and his colleagues. YB-1 mRNA levels have been found to correlate inversely with class II mRNA levels, which suggests that YB-1 may be a negative regulatory protein. YB-2 appears to interact with YB-1 to prevent YB-1 from binding to the class II promoter region. The binding of antigenic peptides to the class II molecules has been studied using photoaffinity probes, and the site of the class II promoter molecules labeled by one such photoaffinity probe has been identified. This site is relatively hydrophobic, is in close proximity to the antigen-binding cleft, and is formed by the first halves of the second domain of the α- and β-chains. In addition, one region of the influenza hemagglutinin molecule that is important for antigen binding and T cell recognition has been identified.

Investigator Robert R. Rich, M.D. (Baylor College of Medicine) and his colleagues have studied structure-function relationships in three models of MHC-mediated antigen recognition by human and mouse T cells. Studies of the maternally transmitted antigen (Mta) in mice suggest that the nuclear gene product involved in Mta expression functions as a receptor for a formulated hydrophobic peptide encoded by a mitochondrial gene. Sequencing of human HLA genes associated with an anomalous MHC class II molecule has revealed mutations in two adjacent codons of the DRB gene that may account for the unusual properties of certain DR1 molecules. Finally, studies of peptide–MHC class II interactions have demonstrated that the staphylococcal enterotoxins, which display several extraordinary antigenic properties, exhibit a specific, high-affinity interaction with class II MHC molecules, leading to class II–dependent, but MHC allotype- and isotype-independent, T cell activation.

The laboratory of Investigator Kirsten Fischer-Lindahl, Ph.D. (University of Texas Southwestern Medical Center at Dallas) has continued its analysis of the Mta transplantation antigen, with the aim of

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achieving a molecular definition of a minor histocompatibility antigen. The group had earlier succeeded in identifying a short peptide, encoded in the mitochondrial genome, which is the maternally transmitted component of this antigen. This peptide is presented on the cell surface by a major histocompatibility class I molecule, Hmt. This year several class I genes from the chromosomal region that encodes Hmt were defined, cloned, sequenced, and characterized. One of these, *R4B2*, is an excellent candidate for the *Hmt* gene. Hmt is the first nonclassical class I molecule known to present an identified self peptide.

Experiments in the laboratory of Associate Investigator Richard G. Cook, Ph.D. (Baylor College of Medicine) are focused on the biochemical structure, regulation of expression, and function of the class I MHC alloantigens encoded by the *Q* and *TL* region genes. Northern blot analyses, two-dimensional gel, and amino acid sequence data have revealed that two *Q* region genes encode the Qa-2 antigen family. Some strains express *Q7* and *Q9*, while others express only *Q9*. Certain anti-Qa-2 monoclonal antibodies also were found to induce lymphocyte activation. Analysis of the 5′ region of a *TL* gene suggests that there are both positive and negative regulatory motifs that control the tissue-specific expression of TL antigens.

Assistant Investigator C. Geoffrey Davis, Ph.D. (University of California at San Francisco) and his colleagues are studying the trafficking of class I products of the human MHC. In the past year the laboratory has made advances in their efforts to establish a model system in which to study endocytosis of these surface molecules. Certain aspects of this system have revealed intriguing features of intracellular processing that are also under investigation. Finally, analysis of chimeric molecules has revealed that cytoplasmic domains not only contain signals sufficient for directing surface molecules into the endocytosis pathway but also play a role in determining whether they subsequently recycle to the surface.

The genetic pathways of early lymphocyte development and the aberrancies that result in malignancy are the concern of Associate Investigator Stanley J. Korsmeyer, M.D. (Washington University) and his colleagues. Highly characteristic translocations between unrelated chromosomes frequently occur in malignant cells of human lymphomas and leukemias. This event may juxtapose the genes of immunoglobulin or T cell receptors with new putative cancer genes. One such gene, *Bcl-2*, isolated from human follicular lymphoma, was shown to extend the survival of cells. A deregulated form of *Bcl-2* was introduced into the genetic material of mice. These animals develop B cell neoplasms, providing a prospective role for this translocation in tumor development. Candidates for cancer-promoting genes have been identified in T cell tumors. Approaches are being developed also to characterize large expanses of the human genome to identify disease loci that lie at considerable distances from known genes.

The laboratory of Investigator Owen N. Witte, M.D. (University of California at Los Angeles) deals with two interrelated problems: cell growth regulation and the differentiation of blood cells. The research is focused on two major areas: 1) the development of techniques that will allow growth and manipulation of specific types of blood cells and 2) the investigation of the function of genes found in certain human leukemias, such as Philadelphia chromosome–positive acute lymphocytic leukemia and chronic myelogenous leukemia, in which the *ABL* oncogene is changed by chromosome rearrangement, leading to abnormal growth control. The long-range goal of this research is to increase understanding of the way in which these oncogene products stimulate and regulate abnormal cell growth and to use this information to understand the control of normal cell growth.

Complement is a recognition and effector system that evolved to protect the host from infectious organisms. It accomplishes this task by attaching to the foreign agent and by promoting the inflammatory response. The bound complement components serve as ligands for receptors on peripheral blood cells and tissue macrophages. This process must be carefully regulated in order that components become attached to foreign and not self tissue. Consequently much of the regulation of the complement system centers on controlling activation of complement at the critical step in which the fragments that can attach to cells are generated. The laboratory of Investigator John P. Atkinson, M.D. (Washington University) has been instrumental in identifying and characterizing such regulatory molecules. Specifically, these regulatory and receptor glycoproteins were found to belong to a new multigene family of functionally, structurally, and genetically related molecules whose structural genes are located on the long arm of human chromosome 1.

In the complement system a cascade of serum proteins is activated by foreign antigens and func-

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tions to help clear these antigens. When activated, complement proteins interact with specific receptors on human cells involved in the immune response. Assistant Investigator V. Michael Holers, M.D. (Washington University) and his colleagues are analyzing the function and regulation of expression of these receptors. The group has cloned a number of these receptors in both human and mouse and is currently using recombinant techniques to analyze their function. It is known that complement and these receptors play roles in chronic inflammatory diseases, such as systemic lupus erythematosus and rheumatoid arthritis. Understanding the role of these receptors and developing abilities to modulate them may provide further methods to treat these diseases.

Analysis of a primary antihapten immune response has given new insight regarding the formation and development of memory B cell lineages. It has been possible to construct a model outlining the development of somatic variants from memory B cell lineages. The murine Ly-6 differentiation antigens are associated with activated T cells. Associate Investigator Alfred L. M. Bothwell, Ph.D. (Yale University) and his colleagues have generated mutants in one of these proteins and are examining the consequences of these mutations on T cell activation as well as on the biosynthesis of the protein. The promoters of the two best-characterized Ly-6 antigens are being studied in detail for regulatory elements that effect the tissue-specific or induced transcription. A human Ly-6 homologue, designated CD59, has been cloned. One function of CD59 is to inhibit the complement system by preventing pore formation through inhibiting oligomerization of complement component C9. It may also have a role in T cell activation via CD3.

Investigator Edward J. Goetzl, M.D. (University of California at San Francisco) and his associates have characterized chemical signals that mediate human immunity and hypersensitivity and have identified cellular receptors for some of the signals. One class of signals is generated when immune cells cleave precursor proteins to liberate peptides resembling transmitters of neural impulses. The immune cell receptors for such neuropeptide-like signals differ in specificity from the nerve cell receptors. Dr. Goetzl and his colleagues have isolated the genetic message for one such immune cell receptor and are working to define its structure to elucidate how it differs from the same class of nerve cell receptors at the molecular level.

Assistant Investigator Donald G. Payan, M.D. (University of California at San Francisco) and his collaborators continue to study the molecular mechanisms by which neuropeptides, such as substance P, and multifunctional mediators, such as histamine, transduce their efforts on specific target tissues. Ongoing attempts to isolate the gene for the substance P receptor are now focused on using the polymerase chain reaction and low-stringency hybridization technologies with known tachykinin receptor genes. The work on histamine is concentrated on expanding the initial observation that the H_2 receptor is coupled to two different signaling transduction pathways, depending on the state of differentiation of the responding cell. In addition, collaborative studies have shown that substance P–containing nerve fibers are the route by which specific viruses infect ocular structures in an animal model.

The laboratory of Assistant Investigator Kenneth J. Hardy, M.D., Ph.D. (Baylor College of Medicine) has focused its studies on the molecular mechanisms regulating the potent and clinically relevant human cytokine, interferon-γ (IFN-γ). This potent immunomodulator, which is implicated in the pathogenesis of rheumatoid arthritis, AIDS, and multiple sclerosis, also serves as an excellent molecular model of T cell–specific cytokine gene regulation. In line with its potential role in autoimmune tissue destruction, IFN-γ was shown to be a potent upregulator of its own gene expression by certain human blood cells, while being strongly repressed by other blood cells and their associated cytokines. Molecular genetic studies, utilizing transfection of expression vectors directly into fully differentiated human blood cells, have permitted detailed elucidation of the genetic regulatory elements in the human IFN-γ gene, providing functional proof of a T cell–specific, orientation-dependent, negative regulatory element. From the analyses, a unifying hypothesis was derived that corroborates and extends current notions on cytokine gene regulation.

Investigator Charles W. Parker, M.D. (Washington University) and his colleagues are exploring genetic influences on immunoglobulin E (IgE)-mediated allergy. In mice, two independently segregating genes have been identified that selectively regulate IgE levels. Studies to localize the genes and to elucidate their roles are under way. Hormonal effects in the autoimmune process are also being examined, with emphasis on systemic lupus erythematosus and the mouse hybrid models for this disease.

GENETIC MECHANISMS INVOLVED IN THE GENERATION OF THE ANTIBODY REPERTOIRE

FREDERICK W. ALT, PH.D., *Investigator*

Dr. Alt's laboratory is defining molecular factors involved in the development of antibody-producing cells. A particular focus is the elucidation of molecular mechanisms that control the genomic rearrangement events involved with this differentiation process.

I. Regulation of Recombination Events During Lymphocyte Differentiation.

The amino terminus of immunoglobulin (Ig) heavy and light chains is highly variable and is responsible for antigen binding. The carboxyl termini of heavy chains have a constant amino acid sequence that determines effector activities. The variable region of Ig genes is encoded by multiple germline elements, the V, D, and J segments, which are assembled into complete V(D)J variable-region genes during the somatic differentiation of B-lineage cells. Dr. Alt's laboratory demonstrated that both Ig heavy- and light-chain variable-region gene segments, as well as related gene segments that encode T cell receptor (TCR) variable regions, are assembled by a common system, VDJ recombinase. The constant region of the Ig heavy chain produced by a clonal B cell lineage can be changed as a result of a separate type of recombination event that juxtaposes one of several downstream constant-region genes to the expressed VDJ gene; this event is mediated by a different recombination system, referred to as class-switch recombinase.

Dr. Alt's past studies suggested the specific assembly of Ig heavy- and light-chain or TCR variable-region genes in appropriate cells and stages within lymphoid lineages is effected by modulating accessibility of substrate gene segments to VDJ recombinase. Accessibility was correlated with transcription of unrearranged gene segments. To elucidate controlling elements, Dr. Alt and his colleagues created transgenic mice that carry a hybrid antigen receptor gene minilocus composed of germline TCR variable-region gene segments (V, D, and J) linked to an Ig heavy-chain constant-region gene with or without a DNA segment containing the Ig heavy-chain transcriptional enhancer (Eμ). Transgenic constructs lacking Eμ did not rearrange in any tissue. In contrast, presence of the Eμ segment within the construct dominantly targeted transgenic TCR D to J joining at high frequency in both B and T cells. However, TCR V to DJ joining within the construct occurred only in T cells and was correlated with transcriptional activity of the unrearranged TCR V gene segment. Therefore Dr. Alt's group has demonstrated elements that can control two separate aspects of VDJ rearrangement. The Eμ element (and possibly associated sequences) acts as a dominant recombinational enhancer to initiate lymphoid-specific D to J rearrangement within the transgenic construct; this suggests that enhancer elements associated with various endogenous Ig and TCR loci may act to target those loci for rearrangement. In addition, an element associated with the V segment in the construct provides T cell–specific control of V to DJ rearrangement. During the past year, new transgenic lines have been generated that contain constructs in which various lymphoid enhancer or promoter elements were mutated or exchanged to define their activity precisely with respect to VDJ recombination.

Heavy-chain class-switch recombination allows a clonal B cell lineage to express the same variable (VDJ) region in association with a different constant region. Dr. Alt's group defined transcription units that initiate upstream of class-switch recombination target sequences of four different germline heavy-chain genes, including γ1, γ2b, γ3, and ε. In addition, they have demonstrated that treatment of B-lineage cells with the polyclonal activator bacterial lipopolysaccharide (LPS) induces transcription from the germline γ2b and γ3 promoters, followed by induction of switch recombination to these genes. Conversely, they demonstrated that addition of the T cell factor interleukin-4 (IL-4) simultaneously with LPS inhibits germline γ2b and γ3 transcription and switching but induces germline γ1 and ε transcription, followed by switching to those constant genes. They also demonstrated that precursor B cells inherit a precommitment to switch to γ2b and γ3 but that treatment with IL-4 can alter that commitment, leading to switching to ε even at this early stage. This evidence suggests that class switching may also be controlled by modulating access of different heavy-chain constant genes to a common class-switch recombinase. Accessibility can be modulated by treatment of cells with external agents and correlates with effects on transcription of the target gene sequences. Recently the group found several proteins that bind to germline constant-gene promoter regions and, as a result, may be involved in regulating switching.

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II. Lymphocyte Development.

Mice carrying the severe combined immunodeficiency (SCID) mutation lack both mature T and B cells due to an impairment of VDJ recombination. Dr. Alt's group characterized this defect in detail by analyzing the ability of Abelson murine leukemia virus (MLV)-transformed SCID pre–B cells to rearrange endogenous or introduced Ig variable-region gene segments. Most pre–B lines derived from SCID mice attempt to rearrange endogenous heavy-, but not light-chain, variable-region gene segments. Such lines express high levels of germline heavy-chain V transcripts but do not express high levels of germline κ light-chain transcripts. However, Dr. Alt's group derived rare SCID pre–B lines that did rearrange endogenous κ V gene segments, and all transcribed the germline κ locus (in accord with the accessibility model described above). The vast majority of SCID heavy- and light-chain coding-sequence joins are grossly aberrant, although SCID pre–B lines can form normal coding joins at very low frequency—perhaps allowing for the "leakiness" of the SCID mutation. In contrast, SCID pre–B cells join the recombination signal sequences flanking germline V gene segments at normal efficiency—mechanistically distinguishing coding and signal join formation. SCID pre–B lines that did not attempt chromosomal coding-sequence joining had a relative and selective growth advantage over those that did; this supports the previous suggestion of Dr. Alt's group that the SCID defect may lead to unrepaired chromosomal breakage and cell death in developing lymphocytes.

Dr. Alt's group is attempting to utilize SCID mice as a model system to create a defined immune system. Transgenic mice carrying functionally assembled Ig heavy- or light-chain genes in their germline have been constructed; the particular heavy- and light-chain variable-region genes employed encode an antibody of known specificity. Various downstream heavy-chain constant genes have been incorporated into some of these constructs. Individual Ig heavy- and light-chain genes have been or currently are being introduced into the SCID mice via breeding with appropriate transgenics. The expectation is that germline acquisition of functionally rearranged Ig heavy- and light-chain genes by SCID mice will allow their precursor B cells to bypass the SCID defect and generate a monoclonal B cell population that expresses an antibody of known specificity. Transgenic/SCID mice containing functional heavy-, light-, or heavy- plus light-chain genes should provide a model to study factors involved in precursor B cell differentiation as well as elusive factors involved in later stages of the immune response, including IgD function and control of somatic mutation.

III. Regulation of Lymphocyte Differentiation.

Dr. Alt's group identified a number of novel genes involved in lymphocyte differentiation. One encodes a myosin regulatory light-chain-like protein; this gene is expressed only in Abelson MLV–transformed pre–B cells from adult marrow but not those from fetal liver. Expression of this gene is greatly induced after treatment of normal pre–B cells with the pre–B growth factor interleukin-7 (IL-7). IL-7 treatment of normal pre–B cells causes a rapid induction of N-*myc* proto-oncogene expression without a substantial effect on c-*myc* expression. The N-, c-, and L-*myc* genes encode highly related nuclear proteins likely to be involved, at least in part, in transcriptional regulation. Differential IL-7 induction of N-*myc*, as opposed to c-*myc*, in pre–B cells supports the suggestion that differential expression of *myc* genes may be involved in progression of cells through lymphoid (and other) differentiation pathways. This hypothesis was based on the observation that both N- and c-*myc* are expressed in precursor B and T cells, but only c-*myc* is expressed after the stage when these cells acquire surface receptors. Within the lymphocyte lineage, L-*myc* expression was observed only in tumors representing very immature T cells.

To further define *myc* function, Dr. Alt's laboratory is using transgenic and gene-disruption approaches. Transgenic mice were used to study deregulated *myc* gene expression during lymphocyte differentiation. When subjugated to dominant Ig heavy-chain transcriptional regulatory sequences, both N- and L-*myc* genes caused lymphoid malignancies. In correspondence to transgene expression patterns, N-*myc* tumors were mostly from B lineage cells, whereas L-*myc* tumors were from T lineage cells. B versus T cell-specific expression of N- and L-*myc* transgenes appeared targeted by sequences within the corresponding *myc* genes. Transgenic N-*myc* tumors representing surface Ig-positive B cells express some pre–B cell stage-specific genes and activities (including VDJ recombinase), possibly due to deregulated N-*myc* expression. To define further the relationship between N-*myc* expression and B cell differentiation, Dr. Alt's group developed homologous recombination

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vectors that upon transfection into embryonic stem (ES) or pre–B cell lines allow efficient isolation of variants containing a disrupted N-*myc* gene. Related constructs are now being used to disrupt both N-*myc* copies in these lines. A variety of transgenic mouse strains or cell lines harboring normal or variant (both with respect to expression or structure) N-*myc* genes were created to complement potential mouse or cell lines in which the endogenous N-*myc* genes are disrupted.

Dr. Alt is also Professor of Biochemistry and Microbiology at Columbia University College of Physicians and Surgeons.

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COMPLEMENT SYSTEM

JOHN P. ATKINSON, M.D., *Investigator*

Complement is a recognition and effector immune system that evolved to protect the host from infectious organisms. The biology of the complement proteins that become bound to antigens and the regulatory and receptor glycoproteins with which these attached fragments interact is the central focus of Dr. Atkinson's research. The metabolism of such immune complexes has an important relationship to many inflammatory diseases of humans.

I. Sulfation of the Fourth Component of Complement (C4).

Sulfation of tyrosine residues is a recently recognized biosynthetic modification of many plasma proteins. Effects of this site-specific modification on protein function are not known, but the activity of several peptides, such as cholecystokinin, is greatly augmented by sulfation. C4, a component that attaches to antigens during complement activation, is one of the few proteins in which sites and stoichiometry of tyrosine sulfation have been characterized. Decreased hemolytic activity of nonsulfated C4 was demonstrated and resulted from impaired interaction with complement subcomponent C1s, the protease that physiologically activates C4. Purified C1s was able to cleave nonsulfated C4, but ~10-fold higher concentrations of C1s were required for that cleavage than for equivalent cleavage of sulfated C4. These results, with C4 as a paradigm, suggest that sulfation of tyrosine residues can have major effects on the activity of proteins participating in protein-protein interactions.

II. Regulators of Complement Activation (RCA) Gene Cluster.

A recent advance in which Dr. Atkinson's laboratory has played a major role is the identification of a multigene family of complement regulatory and receptor glycoproteins. In the past year Dr. Atkinson and his colleagues identified two plasma regulatory proteins [factor H (C3b binding) and C4-binding protein (C4b binding)], two receptors (CR1 and CR2), and two widely distributed regulatory membrane proteins [decay-accelerating factor (DAF) and membrane cofactor protein (MCP)]. These proteins are related functionally because they bind the opsonic fragments of complement, C3b and C4b; structurally they are related because they contain, beginning at their amino terminus, multiple copies of an ~60–amino acid cysteine-rich repeat; genetically they are related because their structural genes are closely linked on the long arm of chromosome 1. The laboratory discovered the MCP molecule, cloned and sequenced three of these proteins (CR1, DAF, and MCP), and most recently found that the order of five of these proteins on an 800 kb fragment is MCP-CR1-CR2-DAF-C4bp.

A. *C3b/C4b receptor or complement receptor type one (CR1).* Translation of human CR1 mRNA in a cell-free system and by *Xenopus* oocytes was accomplished. These experiments suggested that a cotranslational modification of CR1 structure occurred, probably involving a proteolytic cleavage event.

The CR1 protein exhibits an interesting structural organization. The entire extracellular portion of the mature receptor is composed of a tandemly repeated amino acid motif or complement control protein (CCP) repeat of 59–72 residues in length, extending for 30 CCPs in the case of the most common CR1 polymorphic form. Four polymorphic variants of human CR1 have been identified at the protein level. Their reduced forms exhibit molecular weights of 220,000, 250,000, 190,000, and 280,000. Classical genetic and molecular studies indicate that each variant is the product of a different allele. Tryptic peptide mapping, mRNA studies, and genomic analysis indicate that these forms could differ by multiples of an internal repeat. The common polymorphic form (M_r 220,000) exhibits an internal repetition that is seven CCPs in length and is reiterated four times from the 1st to the 28th CCP. It is hypothesized that the polymorphic variants are the result of unequal crossing-over within such repeated regions.

In the course of studying CR1, several overlapping cosmid clones were isolated that together carry a CR1-like sequence that encompasses 40 kb and contains at least 10 potential exons. The CR1-like sequence, which may encode an unreported protein, exhibits 95% homology to CR1 at the nucleotide level and 91% homology at the amino acid level and is likely to have arisen by duplication of at least a portion of an ancestral CR1 gene. A comparison between the CR1-like sequence and CR1 indicates specific examples of identical nucleotide substitutions at corresponding sites in the most highly

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homologous portion of CR1, an observation that is reminiscent of mutation and evolution in repeated genes (concerted or horizontal evolution) and supports the hypothesis that the CR1 polymorphic variants were generated by unequal crossing-over in this region.

B. *Decay-accelerating factor.* DAF is a cell surface glycoprotein that protects cells from autologous complement-mediated lysis. DAF is one of the first phosphatidylinositol-linked molecules to be described on human T cells. Low levels of DAF were found to be expressed on a majority of freshly isolated human T cells, and DAF expression rapidly increased after T cell activation by mitogens. Moreover, antibodies to DAF induced T cell proliferation if the cells are costimulated with phorbol esters. T cell mitogenesis was largely dependent on the phosphatidylinositol-linked form of DAF, because removal of DAF by a phosphatidylinositol-specific phospholipase C eliminates anti-DAF-induced T cell proliferation. These studies suggest that DAF on the surface of T cells may not only serve to afford protection from autologous complement but may also function to transmit signals that induce T cell activation.

C. *Membrane cofactor protein of complement.* MCP is a C3-binding glycoprotein with a characteristic, relatively broad, two-band (M_r 63,000 and 55,000) pattern by SDS-PAGE analysis. A rabbit polyclonal antibody was produced to the purified protein, and this reagent was employed to analyze the distribution of MCP on human peripheral blood cells. Flow cytometric analysis indicated that MCP is unimodally present on all platelets, granulocytes, T helper lymphocytes, T suppressor/cytotoxic lymphocytes, B lymphocytes, natural killer cells, and monocytes. It is not present on erythrocytes. The presence of MCP on granulocytes was unexpected, and further evaluation indicated that MCP of granulocytes has both structural and functional differences compared with MCP of platelets and mononuclear cells. To determine further its tissue distribution, surface-labeled human fibroblast, epithelial, and endothelial cells and cell lines were assessed for the presence of MCP by C3 affinity chromatography and by immunoprecipitation. These cells and cell lines all expressed MCP. The wide tissue distribution of MCP supports the concept that this protein is important in the protection of host cells from complement-mediated damage. Also, the expression of MCP was found to be modulated by SV40 transformation of two fetal fibroblast lines. Transformation led to a 5- to 10-fold increase in expression as well as a preferential expression of the lower molecular weight species, suggesting that viral infection can manipulate the regulation of this host regulatory protein.

Biosynthetic and biochemical analyses indicated that the two forms of MCP were similar and that both contained N- and O-linked sugars. Pulse-chase experiments demonstrated approximately equal quantities of two precursor forms, with molecular weights of 41,000 and 43,000. The lower molecular weight precursor chased with a $t_{1/2}$ of 90 min, while the higher molecular weight precursor chased with a $t_{1/2}$ of 30 min. These experiments indicate that the two forms of MCP are structurally similar and are probably derived from two distinct precursors. They also suggest that variations in the rate of processing of two intracellular precursors may account for the differential expression of the mature forms. Molecular analysis of these two precursor and mature forms of MCP should be informative relative to the structural basis for this differential processing of two highly homologous precursor proteins.

Dr. Atkinson is also Professor of Medicine and of Microbiology and Immunology at Washington University School of Medicine and Physician at Barnes Hospital, St. Louis.

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MECHANISM OF LYMPHOCYTE DIFFERENTIATION

ALFRED L. M. BOTHWELL, PH.D., *Associate Investigator*

There are three areas of research in Dr. Bothwell's laboratory: 1) analysis of the function and structural properties of a family of T cell differentiation antigens, designated Ly-6; 2) molecular characterization of regulatory sequences controlling Ly-6 gene transcription; and 3) study of the development of a model T cell–dependent immune response, especially with regard to the process of somatic mutation and development of cell lineages.

I. Molecular Analysis of Structural and Functional Properties of Ly-6 Antigens.

Several experiments have implicated Ly-6 antigens in lymphocyte activation. Mitogen stimulation of peripheral lymphocytes causes high levels of Ly-6 expression of both B and T cells. Perhaps the most intriguing observation is that monoclonal anti-Ly-6 antibodies can activate splenic T cells or antigen-specific T cell hybridomas to proliferate and release interleukin-2 (IL-2). Expression of the T cell receptor on the cell surface is necessary for activation by monoclonal antibodies. In addition, mutants of T cell hybridomas that have lost the normal levels of Ly-6 can no longer respond to the appropriate antigen. These results suggest that these antigens can either amplify or modify physiologic signals generated by elements of the T cell receptor complex or are involved in a distinct pathway for T cell activation.

Two of the Ly-6 antigens, Ly-6C and Ly-6A/E, have been characterized very extensively. The polypeptides are ~14–18 kDa and have extensive disulfide bonds. The proteins are anchored in the cellular membrane by a phosphatidylinositol (PI) lipid linkage. Dr. Bothwell's laboratory is attempting to elucidate the function of these molecules. A mutational analysis of the protein is being performed. Initially, the carboxyl terminus has been mutated to create a transmembrane form of this antigen. This analysis has revealed that there are at least two signals involved in the biosynthesis of a PI-linked protein. During its biosynthesis, ~30 amino acids are removed from the carboxyl terminus and the PI linkage is attached. There is a minimal length of the carboxyl terminus that is required, and a second more complex signal is required near the point of proteolysis.

A transmembrane form of the protein has been generated by appending the transmembrane and cytoplasmic tail of a class I antigen to the Ly-6 structure. These constructs are being introduced into T cell clones to assess their effect on activation. Another approach to alter normal Ly-6 function is to knock out the expression of the gene, using antisense transcripts or deletion of the gene by homologous recombination. Both experiments are in progress.

A potential human homologue of Ly-6 was identified by others as a PI-linked protein on erythrocytes and designated MEM-43 or CD59. This protein has also been studied as an inhibitor of complement function. It appears to inhibit the oligomerization of C9. In other experiments it has been characterized as an antigen that is expressed on accessory cells and necessary for T cell activation via CD3. The cDNA was isolated and shown to have structural homology to Ly-6 antigens. In contrast to murine Ly-6, the human CD59 is found as a single gene on chromosome 11. It is possible that the murine Ly-6 antigens have an analogous function in mice. The high level of Ly-6A in the kidney might function to protect cells from high levels of immune complexes. The possibility that Ly-6 may bind to murine C9 is being examined.

II. Regulation of Ly-6 Gene Transcription.

The Ly-6A/E and Ly-6C genes and their promoters are being analyzed in detail. These two genes are inducible with interferon-α,β and interferon-γ, yet the DNA sequence of these promoters does not reveal the consensus GA box found in all other interferon-α,β–responsive genes. A deletion analysis of both of these promoters is being accomplished by deriving stably transformed clones of BALB/3T3 cells. An attempt to analyze the interferon responsiveness with transient transfections using chloramphenicol acetyltransferase (CAT) vectors was unsuccessful. However, transient CAT assays have been used to identify other elements of the Ly-6A/E promoter. At present, the α,β as well as the γ DNA elements reside within ~1.5 kb 5′ of the start site for transcription. Deletions located about every 200 bp within these regions are being analyzed.

Two autoimmune strains of mice, non-obese diabetic (NOD) and NZB, have a common rearrangement in the promoter of the Ly-6C gene. This rearrangement is not present in the progenitor of NOD, the NON strain. Genomic DNA clones of the NOD

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Ly-6 genes are being analyzed to determine the nature of the expressed gene. Other investigators have shown aberrant Ly-6C expression in T cells from NOD pancreatic islets.

III. Analysis of the Development of an Antibody Response.

The cellular basis for heterogeneity in the secondary immune response was studied earlier by creating a large set of B cell hybridomas from a single C57BL/6 mouse immunized with the hapten NP coupled to chicken gamma globulin. Individual progenitor cells were identified by the uniqueness of the DNA sequences of the heavy- and light-chain joining regions, which are established early in the life of a B cell. The hybridomas were also used to characterize somatic mutation, affinity, and the presence of certain antigenic determinants on these antibodies. The results demonstrated the extreme oligoclonality in the normal secondary response and suggested that the oligoclonality seen in the secondary immune response would also be seen in the primary immune response to NP.

Evidence for oligoclonality was found in the primary anti-NP response, but the analysis of the expressed sequences revealed much more about the early events in memory formation. At day 12, there were seven predominant clones detected. Three of these clones expressed the prototype Ig gene combinations. Surprisingly, all members of a family expressed the same heavy-chain isotype and possessed only common somatic point mutations. For the prototype combination, the most common single point mutation that creates a 10-fold higher affinity antibody was observed in two of three families.

These results reveal the characteristics of the onset and development of memory B cells. For the first time it is possible to develop a model for this process. The strength of the response, in part due to the affinity of the germline-encoded antibodies, has revealed the affinity threshold for selection of these antibodies. Once a successful mutation occurs in this response, a single memory B cell must differentiate into a plasma cell. It then either reduces considerably or terminates the occurrence of additional somatic mutation. The resultant expansion of the plasma cell lineage generates a wave of B cells that are seen as a family of related antibodies. The mutational mechanism must initiate early, probably by day 3–4, and is a continuous process in memory cells. The original germline sequence is not maintained, because increases in affinity are selected. In this response, additional mutations must gradually accumulate, because by three weeks considerably more mutations are evident.

Dr. Bothwell is also Associate Professor of Immunobiology and Biology at Yale University School of Medicine.

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$CD4^+$ T CELL HETEROGENEITY

KIM BOTTOMLY, PH.D., *Associate Investigator*

$CD4^+$ T cells have been shown to be functionally heterogeneous, with $CD4^+$ T cells directing both humoral and cell-mediated immunity. Because of the diverse capabilities of CD4-bearing T cells, Dr. Bottomly became interested in determining if a single cell could mediate all functions characteristic of $CD4^+$ T cells or if specialized subsets existed within the $CD4^+$ T cell population. Two approaches were used in the initial studies. First, monoclonal antibodies were shown to identify phenotypic differences between CD4-bearing T cells, and these phenotypic differences correlated with functional differences. Studies in both the rat and human systems showed that $CD4^+$ T cells could be divided into naive and memory cells on this basis. Second, mouse monoclonal T cells showed differences in their functional capabilities, and this correlated with the cytokines they released. Th1 cells are cloned T cells that release interleukin-2 (IL-2), interferon-γ (IFN-γ), and lymphotoxin (LT); these cells also mediate delayed hypersensitivity responses, activate macrophages, and kill class II major histocompatibility complex (MHC)-bearing B cells. Th2 cells are cloned T cells that release interleukin-4 (IL-4) and interleukin-5 (IL-5) and help B cells, eosinophils, and mast cells. Further studies comparing these two approaches have pointed to inconsistencies or differences in the defined $CD4^+$ subsets. To resolve these differences and to investigate functional equivalents of Th1 and Th2 cells *in vivo*, this laboratory has produced a monoclonal antibody that distinguishes between cloned lines of Th1 and Th2 cells. This antibody has been used to separate normal CD4 T cells to study their function and activation requirements.

I. Characteristics of Subsets of $CD4^+$ T Cells.

Initial studies using the monoclonal antibody 16A showed that CD4 T cells from normal mice could be accurately subdivided into stable populations. When the density of the determinant was used as a marker, 16A high-density cells produced IL-2 and IFN-γ upon activation with T cell mitogens in short-term culture; whereas the 16A low-density cells produced IL-4 and IL-5 and provided excellent helper T cell function. Thus it appears that 16A monoclonal antibody divides normal CD4 T cells into Th1-like and Th2-like T cell subsets. By immunoprecipitation, 16A was shown to bind to common leukocyte antigen or CD45. This is of particular interest in that the majority of other antibodies that subset CD4 T cells in other species are also directed against CD45. Dr. Bottomly and her colleagues compared the subsets defined by 16A in the three species with subsets defined by other anti-CD45 antibodies.

In the rat and human, CD4 T cells are subdivided by anti-CD45 antibodies into naive and memory T cells. To examine whether 16A separates comparable populations, Dr. Bottomly and her colleagues tested 16A high- and low-density cells for their ability to make memory or recall responses to a specific antigen. Recall T cell activation, as measured by cytokine release, indicated that both subsets contain memory T cells. Thus subsets defined by 16A are not equivalent to those subsets defined in the rat and human. A second possibility suggested by the data is that 16A subdivides memory T cells into Th1-like and Th2-like cells. Either their immediate precursor is uncommitted functionally and becomes committed upon contact with antigen/antigen presenting cells or their immediate precursor is precommitted and upon differentiation becomes a Th1 or Th2 cell. The latter possibility seems unlikely, in that cloned T cell lines derived from 16A-high cells that produce IL-2, switch to the production of IL-4. Thus it would appear that 16A-high cells can give rise to 16A-low cells, phenotypically and functionally. Finally, a study of the phenotype and function of 16A-high and 16A-low cells over time after activation with antigen suggests that the main activity of 16A-high cells is to release IL-2, clonally expand, and give rise to 16A-low cells. By contrast, the main activity of 16A-low cells is to release IL-4, IL-5, and IFN-γ upon stimulation. Because most helper activity and cytolytic activity is found in this population, they may be considered T effector cells. Thus the data suggest that 16A separates CD4 T cells into a T proliferator population and a T effector population.

II. 16A Defines Two Subsets of Memory T Cells.

Analysis of the proliferative capabilities of 16A-defined subsets of CD4 indicates that 16A-high cells give the best recall T cell proliferative response, which decreases over time. This proliferative response is blocked by anti-IL-2 antibodies. 16A-low cells have a limited proliferative potential early after

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restimulation, which is partially inhibited by anti-IL-2 and partially by anti-IL-4 antibodies. This proliferation is sustained and becomes more IL-4 dependent over time. This suggests that most immediate-recall clonal expansion occurs when a cell is 16A high density. Analysis of the helper T cell function of the two subsets of CD4 T cells indicates that hapten-specific helper activity is predominantly in the 16A-low population. Primed 16A-low cells assist very well for IgM and IgG1 responses and less well for IgG2a and IgG2b in the time frame tested. These findings are consistent with the known helper function of the cytokines IL-4, IL-5, and IFN-γ, which are produced by 16A low-density CD4 T cells.

The question of where Th1 and Th2 cells fit within the 16A subsets still remains. Because 16A-low cells produce cytokines characteristic of Th1 and Th2, the progeny of individual cells (short-term cloned lines) derived from the 16A population were tested for cytokine release upon stimulation with antigen. Some of the cloned lines released IL-4 and IL-5 but not IL-2 or IFN-γ; these lines are similar to Th2 cells, as originally defined. Some of the cloned lines released IFN-γ but not IL-4 and IL-5; these lines are similar to Th1 cells. Some monoclonal T cells released all three cytokines, suggesting an intermediate phenotype. These data suggest that the 16A-low population is further subdivided into Th2 and Th1 cells and perhaps other phenotypes.

In the future, Dr. Bottomly will examine how 16A-low cells mature into the equivalent of Th1 and Th2 cells and to what extent intermediate forms exist *in vivo*. This is of great importance in protective immunity, as has been shown by studies of leishmaniasis and leprosy.

III. CD45 Isoforms May Be Predictive of Functional Capabilities of a CD4 T Cell.

Previous studies have shown that most of the antibodies used to subset CD4 T cells recognize CD45. An analysis of the specificity of binding was undertaken, to determine whether 16A was similar or identical to other anti-CD45 antibodies. CD45 molecules are quite heterogeneous in terms of their molecular weight, partly because of differences in glycosylation and partly because of differential splicing of three variable exons encoding an extracellular, amino portion of the molecule. By differential splicing, one gene can give rise to eight predicted isoforms. By transfecting cDNAs representative of several of the isoforms, it is possible to determine which isoform is required for antibody binding. In this way it was shown that the second variable exon is required for 16A reactivity, and therefore 16A does not bind to all possible CD45 isoforms. This antibody differs from those directed at the human CD45 molecule, which may explain the differences seen between species. Because 16A discriminates between IL-2 and IL-4 producers in normal CD4 populations and between cloned lines of Th1 and Th2, functionally distinct cells may express characteristic isoforms of CD45. These isoforms can be identified either by monoclonal antibodies that distinguish between isoforms or by polymerase chain reaction analysis of mRNAs encoding different isoforms. By both types of analysis, Th2 cells express the high-molecular-weight isoforms of CD45, including the three-variable exon form, whereas Th1 cells express only the low-molecular-weight forms, especially the no-exon form. This dramatic difference in the CD45 phenotype of these functionally distinct panels of Th1 and Th2 cells suggests that shifting of CD45 isoforms is predictive or a result of functional differences between CD4 T cells.

Future studies will focus on the developmental relationship between these CD4 subsets and the mechanism by which a proliferating cell differentiates into an effector cell and switches the panel of cytokines released. In particular this laboratory will focus on understanding the mechanism by which the immune response selects the effector mode that is dominant in a particular immune response.

Dr. Bottomly is also Associate Professor of Immunobiology at Yale University School of Medicine.

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MOLECULAR GENETICS OF THE HLA AND CYTOKINE SYSTEMS

DAVID D. CHAPLIN, M.D., PH.D., *Associate Investigator*

During the past year investigations in this laboratory have focused on two principal areas: 1) analysis of the structure of the human major histocompatibility complex (MHC) and 2) characterization of the interleukin-1 (IL-1) family of molecules.

I. Structure of the Human MHC.

A. *General characteristics.* The MHC (in humans designated the HLA complex) is one of the best characterized genetic regions in higher vertebrates. It spans $3-4 \times 10^6$ bp of DNA and contains at least 20 functional genes. The complex is of interest not only because of the pivotal role of its gene products in many immunological reactions but also because it is one of the largest and best characterized mammalian gene clusters. Although in general its gene products display related functions, selected genes or subsets of genes show discrete regulation, both in terms of the developmental and tissue-specific control of their expression and of the inter- and intracellular mediators that modulate their expression.

The organization of the cluster has been generally conserved in all vertebrates studied to date. The complex can be divided into three regions that encode distinct classes of molecules. The class I and II genes encode structurally related polymorphic cell surface glycoproteins that are required for antigen presentation and lymphocyte-mediated killing of virally infected cells. Primary structural analyses indicate that the class I and class II genes have evolved by duplication of a common ancestral gene sequence and comprise two limbs of the immunoglobulin supergene family. The class III region, characterized in detail by this laboratory, encodes apparently unrelated molecules with diverse functions, including activation of the blood complement system, biosynthesis of adrenal steroid hormones, and regulation of nonspecific inflammatory reactions. Additional genes with unknown functions have recently been identified within this portion of the MHC.

B. *Polymorphism and linkage disequilibrium.* Most of the genes within the MHC show a high degree of polymorphism. Certain alleles are found to be present with marked linkage disequilibrium. In Caucasians, ~30% of all HLA haplotypes are found as linkage disequilibrium groups (or extended haplotypes). These extended haplotypes are particularly important because they have been shown to be associated at high frequency with a large number of human disease states.

C. *Physical characterization of the HLA complex.* Analyses of the evolution, genetics, and disease associations of the MHC remain hampered by the current incomplete knowledge of the structure and composition of the full complex. Although the human MHC has been extensively studied, only approximately half of the complex has been analyzed at the molecular level. The remaining half has been mapped only at low resolution, using long-range restriction fragment analysis.

To obtain the physical substrate to analyze the MHC at high resolution, Dr. Chaplin's laboratory is isolating genomic clones spanning the entire complex. Because of the large size of the MHC, emphasis is being placed on yeast artificial chromosome (YAC) vectors for isolation of very large molecular clones. The feasibility of this cloning effort has been demonstrated. Twelve YAC clones containing HLA sequences have been isolated. They average ~270 kb in length and together represent more than half of the predicted structure of the MHC. Individual clones establish for the first time physical linkages of certain loci. For example, one YAC of 210 kb contains both the class I HLA-B and -C loci and establishes that they are separated by ~100 kb. Another clone links the class II HLA-DQα, -DQβ, -DRα, and -DRβ loci. Future studies will focus first on completion of the YAC map and on high-resolution analysis of the structure of each YAC clone. Subsequently this mapping data will be applied to a comparison of the structures of different MHC extended haplotypes and to a search for unrecognized genes within the newly cloned regions.

II. Characterization of Mouse IL-1.

Molecular and cell biological analyses first identified two forms of IL-1 in humans. Although the genes encoding these two forms (IL-1α and IL-1β) share only ~65% nucleotide sequence identity, the protein products they encode show identical receptor binding affinities and indistinguishable biological activities. Both molecules are synthesized as ~31 kDa intracellular promolecules and are found extracellularly as carboxyl-terminal 17 kDa processed fragments. Neither promolecule contains a

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conventional signal peptide, so the mechanism of release from the cell is unknown.

Two IL-1 genes have also been identified in the mouse, and their evolution by duplication and divergence from an ancestral IL-1 gene has been established; however, the protein product of only the IL-1α gene has been analyzed.

A. *Characteristics of recombinant mouse IL-1β.* To obtain sufficient IL-1β for preparation of antibodies, Dr. Chaplin and his colleagues expressed the protein in *Escherichia coli.* When the expression vector pMON5743 (courtesy of Dr. Peter Olins, Monsanto Company) was used, recombinant 17 kDa IL-1β represented nearly 20% of the total bacterial protein. High biological potency was shown, with specific activity equal to that of mouse IL-1α, as determined using the thymocyte coproliferation assay. In *E. coli*, 17 kDa IL-1β was targeted to the bacterial periplasmic space. Periplasmic targeting is analogous to mammalian protein secretion, being dependent on a prokaryotic signal peptide equivalent. For 17 kDa IL-1β, periplasmic localization was seen in the absence of any recognized eukaryotic or prokaryotic secretion signal peptide sequences. Periplasmic targeting was not observed with either the 31 kDa pro-form of IL-1β or the pro- or mature forms of IL-1α. The molecular basis for the differential targeting behavior of recombinant 17 kDa IL-1α and IL-1β remains unknown; however, it suggests that the mechanism of release of IL-1β from its normal cellular source may be different from that of IL-1α.

B. *Preparation of anti–mouse IL-1β antibodies.* Rabbits and Armenian hamsters were immunized for the production of anti-IL-1β antibodies. All antisera and monoclonal antibodies produced by immunization in Freund's adjuvant recognized only denatured IL-1β and were ineffective at neutralizing IL-1β biological activity, suggesting that the epitopes of IL-1β characteristic of the biologically active molecule are particularly sensitive to denaturation. In contrast, when animals were immunized with IL-1β in alum, strong neutralizing antisera were produced in rabbits. Hamster monoclonal antibodies are now being produced using similar protocols.

Initial analysis of IL-1 produced by lipopolysaccharide-stimulated mouse peritoneal macrophages (PECs) using the neutralizing rabbit antiserum indicated that IL-1β represents approximately half of the total IL-1 produced by PECs. Pulse-chase labeling experiments showed that the kinetics of biosynthesis of mouse IL-1α and IL-1β were similar. More than 90% of newly synthesized IL-1α and IL-1β is degraded intracellularly, with very little IL-1 released from the cell. This raises the possibility that, in addition to its role as an immunomodulatory cytokine, IL-1 may have other specific intracellular functions.

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CHEMISTRY AND REGULATION OF EXPRESSION OF MAJOR HISTOCOMPATIBILITY COMPLEX–ENCODED CLASS I ANTIGENS

RICHARD G. COOK, PH.D., *Associate Investigator*

The research in Dr. Cook's laboratory is focused on the biochemical structure, mechanisms responsible for tissue-specific regulation of expression, and function of class I cell surface antigens encoded within the *Q/TL* region of the murine major histocompatibility complex (MHC). Approximately 30 class I genes have been defined in the MHC, and more than 20 are localized to the *Q/TL* region, which is telomeric to the major transplantation antigen loci *H-2K,D,L*. Unlike the classical transplantation antigens that bind foreign peptides and serve as restriction elements for the recognition of antigen by specific T cells, biological functions for the Qa and thymus leukemia (TL) class I antigens have, for the most part, remained elusive. All have been shown to serve as weak transplantation antigens, and several display a limited tissue distribution in comparison with the H-2K,D,L antigens. Although most of the class I genes lie within the *Q/TL* region, only a handful have been demonstrated to encode the serologically defined products that reside in this region. It is likely that the Qa and TL antigens also bind foreign and self peptides and thus function in a manner similar to H-2K,D,L, but there is little evidence for this. Recent data suggest that the novel γδ-receptor T cells may recognize or be restricted to antigens encoded within the *Q/TL* region. Thus these molecules may subserve a specific and special function in immune recognition and surveillance. Several of the class I genes in this region are thought to be nonfunctional pseudogenes, whereas others may be functional and encode products that have eluded detection. Dr. Cook and his colleagues are investigating the function, structure, and regulation of expression of the Qa-2 antigen family, the Qa-1 and TL alloantigens.

I. *TL* Region–Encoded TL Antigen.

Although 13–18 class I genes are within the *TL* region, only one serologically and biochemically defined product, TL, has been correlated with a specific gene. The *T13* gene has been shown to encode a TL antigen in the BALB/c strain. Several of the other *TL* genes are pseudogenes; others appear to be functional and may encode products that have not yet been defined.

TL displays a more restricted tissue distribution than other class I antigens. It is expressed on thymocytes, certain leukemia cell lines, and activated peripheral T lymphocytes. Other tissues, including resting peripheral T cells, B cells, and macrophages, are negative. Recent data from another laboratory indicate that a TL-like molecule and the *T13* gene product are expressed in fetal liver cells. Because of this distinctive regulation of TL expression, Dr. Cook and his colleagues are attempting to characterize the molecular genetic controls responsible for *TL* gene expression and activation. Previous data have shown that the *TL* message is detected in thymocytes and activated T cells but not in resting peripheral T cells; this agrees with the serological and biochemical studies. For thymocytes from any given *TL* haplotype, there were 2–3 *TL* mRNA species differing in size. In contrast, *TL* transcripts from activated T cells were less heterogeneous and represented a subset of the transcripts seen in thymocytes. Thus activated T cells may express fewer *TL* genes or may contain fewer aberrant transcripts. The 5′-upstream region of the *T13* gene is being analyzed for enhancer elements that may be important for the novel cell lineage and activation-state expression of the *TL*. Various 5′ segments fused to the reporter gene chloramphenicol acetyltransferase (CAT) have been electroporated into several cell lines and tested for CAT activity. Preliminary data indicate that there are enhancer elements within the *T13* upstream region that seem to function in a tissue- and cell-specific manner. Experiments to define more precisely these enhancer motifs and the nuclear factors responsible for regulated expression of *TL* are in progress.

II. Murine Qa-2 Antigen Family.

An additional *Q/TL* region molecule being examined in this laboratory is the Qa-2 antigen family. Cell surface forms of the prototype Qa-2 antigen (which was presumed to be nonpolymorphic) and the Qa-6 antigen are indistinguishable by biochemical techniques; however, based on analysis of recombinant mouse strains, Qa-6 is encoded or controlled by a gene distinct from the *Qa-2* gene. The Qa-6 determinant does not appear to result from a modification of the Qa-2 antigen.

Dr. Cook's laboratory has correlated the expression of Qa-6 with a distinct Qa-2–associated polypeptide found in Qa-2^+6^+ but not Qa-2^+6^- strains.

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This biochemical heterogeneity in Qa-2 molecules is detected after removal of N-linked oligosaccharides from cell surface forms or by analysis of precursor intracellular forms of Qa-2. Gene transfection studies from this and other laboratories and amino-terminal amino acid sequence analyses of Qa-2–reactive proteins have documented that at least two genes, *Q7* and *Q9*, are normally expressed in cells and encode Qa-2 antigens. Biochemical studies on the *Q* region gene transfectants and Northern blot analyses indicate that the *Q7* gene encodes the Qa-2 molecules that express the Qa-6 determinant. Thus those strains that are Qa-2^+6^+ express both the *Q7* and *Q9* genes, whereas those strains that are Qa-2^+6^- express only the *Q9* gene.

The Qa-2 cell surface molecules, like several other antigens, are not integral membrane proteins but are attached to the cell surface by a glycolipid anchor. Many of these glycolipid-attached proteins have the capacity to induce cell activation and proliferation after the binding of specific antibodies to these proteins. Dr. Cook's laboratory has developed a panel of anti-Qa-2–specific monoclonal antibodies and examined them for the ability to activate Qa-2^+ cells. Several can induce cell proliferation when a second accessory signal is also added. The extent of cell activation induced by these anti-Qa-2 monoclonal antibodies seems to correlate with the cell surface density of Qa-2; high levels of Qa-2 lead to a more vigorous proliferative response. These data suggest that the Qa-2 antigens may play a role in normal cellular activation processes. Qa-2 may serve an accessory role in the normal T cell receptor antigen-specific activation pathway or may serve as a receptor for cell-associated or soluble ligands important in novel activation or cell-to-cell interaction pathways. Experiments are in progress to characterize the regions of the Qa-2 molecule and accessory signals that are essential for inducing cell activation as well as the intracellular events and second messengers involved in the Qa-2 activation pathway.

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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 differentiation of hematopoietic stem cells into immunocompetent T and B lymphocytes is explored. A major goal is to define abnormalities in these pathways of cellular differentiation that lead to immunodeficiencies and lymphoid malignancies.

The accessibility of the avian embryo makes it a convenient model for developmental studies. The existence of separate T and B cell lineages with a common hematopoietic stem cell origin was elucidated through comparative studies of immune system development in chickens and mammals; this compartmentalization of the immune system is seen in all vertebrates. The genes encoding antibody products of B cells are remarkably similar throughout the vertebrate kingdom, although divergent strategies exist for generating antibody diversity.

I. B Cell Development.

B cells derived from a single IgM^+ precursor in bursal follicles were observed to produce a diverse spectrum of immunoglobulin light and heavy chains, which supports genetic evidence for somatic diversification of the avian B cell repertoire.

Epstein-Barr virus transformation of human pre–B cells was used to identify a novel cell type characterized by expression of immunoglobulin κ or λ light chains without heavy chains. The heavy-chain (IgH) gene loci of κ-only clones were in germline context or had undergone only D-J_H rearrangement. This suggests the μ-chain protein is not an obligatory prerequisite for initiating light-chain gene rearrangement.

Examination of the IgH gene configuration in a large panel of human B and T cell malignancies indicated that the recombinase activity involved in immunoglobulin isotype switching is primarily confined to B lymphocytes, often ineffective, and lacks isotype specificity.

II. T Cell Development.

Information on the T cell receptors and their encoding genes is available for mammals, and recent studies in Dr. Cooper's laboratory indicate remarkable parallels in avian T cell development. Monoclonal antibodies to chicken T cell antigens were used to trace the sequential development of T cells expressing γδ-like T cell receptors (TCR1) and of cells expressing αβ-like T cell receptors (TCR2). Both the TCR1 and TCR2 heterodimers reach the surface with a complex of CD3-like proteins. The subpopulations of avian T cells that express these receptors closely resemble their mammalian counterparts.

The monoclonal antibodies against chick TCR1 and TCR2 were the first TCR isotype-specific antibodies that could be used to identify native TCR1 and TCR2 molecules on viable T cells. The experimental exploitation of these monoclonal antibodies revealed several interesting features of T cell development, especially regarding the enigmatic γδ T cell. Developing as a separate sublineage, TCR1 cells are generated before TCR2 cells. From the time they emerge in the embryonic thymus, TCR1 cells express relatively high levels of their receptor complex, whereas TCR2 cells express gradually increasing levels of their surface receptors as they undergo maturation. The transit time of TCR1 cells through the thymic cortex, into the medulla, and out to peripheral lymphoid tissues is several days less than that of TCR2 cells, and the homing patterns of the two are distinctive. TCR1 cells migrate preferentially to splenic sinusoidal areas and intestinal epithelium, while TCR2 cells locate primarily in the splenic periarteriolar areas and intestinal lamina propria. In thymus and the circulation, most TCR1 cells lack both the CD4 and CD8 accessory molecules, but those in peripheral lymphoid tissues express CD8. This contrasts with the well-known pattern of CD4 and CD8 expression by TCR2 thymocytes before they mature into $CD4^+$ helper or $CD8^+$ cytotoxic T cells. The TCR1 subpopulation is surprisingly large in birds, constituting up to 50% of the adult T cell pool. This peripheral pool of TCR1 cells can be severely limited by early thymectomy, apparently because of a limited capability for population expansion.

These observations indicate phylogenic conservation of the TCR1 subpopulation of cells. The preferential homing of the TCR1 cells to splenic sinusoids and intestinal epithelium places them in a strategic defense position. Their expression of CD8 in these sites implies a cognitive kinship with cytotoxic TCR2 cells that utilize the CD8 accessory molecule to reinforce their recognition of antigenic peptides present in the groove of major histocom-

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patibility complex (MHC) class I molecules. The avian data also suggest that TCR1 cells are not subjected to the intrathymic selection pressures that mold the TCR2 cell repertoire. Failure to eliminate potentially autoreactive TCR1 clones in the thymus may suggest an immunoregulatory role for this subpopulation of T cells.

A remarkable finding is the generation of a third sublineage of avian T cells that develops after the TCR1 and TCR2 cells. The TCR3 receptors resemble TCR2, but one of the two receptor chains is smaller and has a distinct peptide composition. A monoclonal antibody against TCR3 fails to recognize TCR1 and TCR2, and vice versa. Intrathymic development of TCR3 cells parallels that of TCR2 cells, in that surface receptor expression increases gradually during the maturation process, which involves simultaneous CD4 and CD8 expression before differentiation into single $CD4^+$ or $CD8^+$ cells. Although the avian TCR genes are not cloned yet, the available biochemical and biological evidence suggests that TCR2 and TCR3 represent αβ subclasses and implies the sequential utilization of different sets of β-chain genes. This conclusion, if correct, may predict an analogous developmental sequence in the generation of the αβ TCR repertoire in mammals.

Chick-quail chimeras, created by embryonic transplants of thymus or other lymphoid organs, were used to make several interesting observations. The first wave of progenitor cells that enter the thymus gives rise to all three T cell sublines. The TCR1, -2, and -3 sublines are generated exclusively in the thymus but may recirculate from the periphery to the thymus medulla. A third lineage of lymphocytes, which express a CD3 antigen in the cytoplasm and CD8 on the surface, develops independently of the thymus and the bursa. The nature and evolution of these TCR0 cells are being examined.

III. IgA Receptor.

A surface receptor for IgA_1 and IgA_2 antibodies has been identified on monocytes and macrophages. This M_r 62,000 molecule has been purified, and its molecular characterization is in progress.

Dr. Cooper is also Professor of Pediatrics, Medicine, and Microbiology at the University of Alabama at Birmingham.

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TRAFFICKING OF MAJOR HISTOCOMPATIBILITY COMPLEX CLASS I MOLECULES

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Studies in Dr. Davis's laboratory have focused on the trafficking of molecules of the human major histocompatibility complex (MHC), with emphasis on endocytosis. The primary approach has been to analyze the behavior of mutant and chimeric molecules expressed in cell lines. These analyses have also elucidated the role of structural features of other cell surface molecules in endocytosis and recycling.

I. Endocytosis of MHC Class I Molecules.

Endocytosis of class I molecules in activated T lymphocytes has been reported by other laboratories. However, there have been no insights into its functional consequences. One reason for this has been the inability to dissect endocytosis from other processes associated with T cell activation. To circumvent this difficulty, Dr. Davis and his colleagues have constructed chimeric molecules containing the extracellular domain of the human HLA molecule A2.1 and cytoplasmic domains from other molecules that endocytose either constitutively or in a regulated fashion. This promising approach has met with some difficulties.

Most attention to date has been devoted to chimeras in which either the cytoplasmic domain alone or the cytoplasmic and transmembrane domains together are derived from the low-density lipoprotein (LDL) receptor, a membrane protein that is endocytosed and recycled constitutively. Although these chimeras were readily expressed and behaved as anticipated when transfected into COS cells, repeated attempts at isolating stable transfectants (including the closely related CV-1 cells) expressing these molecules on their surface were unsuccessful. In several cases it was possible to immunoprecipitate from transfected cells biosynthetically labeled A2.1 heavy chains that had not associated with β_2-microglobulin and appeared to be retained in the endoplasmic reticulum. These results, together with the COS cell results, indicate that the chimeric constructs are capable of coding for transmembrane proteins but that the transport of these proteins out of the endoplasmic reticulum is blocked in many cell types. Expression of full-length A2.1 as well as an array of site-specific mutants in the same cell lines has encountered no such difficulties.

In parallel, Dr. Juerg Baenziger has undertaken to develop a technique to expedite the expression of these constructs in a variety of cell lines. Accordingly, he has cloned three constructs, including the full-length A2.1 cDNA, a tail-minus A2.1, and an A2.1/LDL receptor chimera, into vaccinia expression vectors. Although infection of CV-1 cells with the recombinant vaccinia resulted in high levels of expression of all three constructs, all three were retained in the endoplasmic reticulum. This pattern was also observed after infection of two human cell lines. A third human cell line that lacks endogenous class I molecules allowed readily detectable surface expression of the three constructs.

Although the establishment of a model system for studying the function of class I molecule endocytosis has been challenging, the final product may provide important information on the synthesis and assembly of these molecules. The recent finding that peptide binding plays a role in the maturation of nascent class I molecules may be relevant to these studies, and this possibility is being explored. The question of whether these observations are specific to class I molecules that transport slowly is also being examined.

II. Processing of MHC Class I Molecules.

Molecules of the A2.1 haplotype are processed and transported at an extremely slow rate compared with most other cell surface molecules. The half-time for attaining resistance to endoglycosidase H, an indicator of transport through the medial Golgi, is ~2 h in both JY lymphoblastoid cells and transfected CV-1 cells. The orientation of the cytoplasmic domain and the fact that this domain is displayed in many different forms in class I molecules as a result of alternate splicing led Dr. Davis to test the possibility that the cytoplasmic tail might contain retention signals that could be transferred to other molecules. Although the initial experiments clearly showed that splicing the A2.1 cytoplasmic domain to the LDL receptor molecule dramatically reduced its transport to a rate similar to that of native A2.1, this result was not reproduced in subsequent transfections. A naturally occurring shortened form of the A2.1 cytoplasmic domain resulting from deletion of exon 7 hinders transport of the LDL receptor significantly.

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III. Signals Mediating Receptor Recycling.

In general, cell surface receptors capable of being internalized can be divided into two classes—those that recycle back to the surface and those that do not. LDL receptors and MHC class I molecules belong to the first class; the T cell surface molecule CD4 belongs to the second. Although it is known that the extracellular domain can be a factor in determining a receptor's fate subsequent to internalization, a possible role for the cytoplasmic domain has not been examined. Dr. Davis and his colleagues have found that splicing the transmembrane and cytoplasmic domains of the LDL receptor to the A2.1 extracellular domain allows recycling, whereas splicing the corresponding domains from CD4 to the extracellular domain of either A2.1 or LDL receptor confers phorbol 12-myristate 13-acetate (PMA)-inducible internalization but does not allow recycling. Thus it appears that the cytoplasmic domain can play a role in determining whether an endocytosed receptor recycles to the surface or is targeted for intracellular degradation, at least in the case of CD4.

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T CELL RECOGNITION AND SELECTION

MARK M. DAVIS, PH.D., *Associate 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, and others seem specifically designated to mobilize other cells, particularly B cells. Both of these activities can be mediated through the same recognition apparatus, the α:β T cell receptor (TCR) heterodimer, in close association with the CD3 polypeptides. A central feature of T cell recognition by the α:β receptor is that antigens are often (if not always) "seen" as peptide fragments complexed with either class I or class II histocompatibility molecules. One goal is to try to understand at a biochemical and structural level exactly what TCRs "see," with what affinities, and whether there are any differences from the way antibodies combine with antigens. Another goal is to use transgenic mice to analyze the selection of specific receptor chains and combinations in the thymus. This analysis should be informative with respect to both positive and negative models of thymic selection of the TCR repertoire and questions of affinity for antigen plus major histocompatibility complex (MHC) and MHC molecules alone. A third goal concerns the nature of the recently described γ:δ TCR found on a minority of mature T cells and on the surface of some T cell precursors. Transgenic systems involving the implanting of specific γδ genes in mice are providing some clues to the role of this antigen receptor in the immune system and their relationship to the α:β-bearing T cells.

The T and B lymphocytes are also important as model systems for cellular differentiation and lineage relationships at a molecular level. Toward this end the isolation and characterization of possible regulatory molecules is useful. Although the studies described here deal largely with one such molecule (the product of the XLR gene family), the technology now exists to isolate other cell-type-specific, nuclear protein genes fairly rapidly.

I. A Model for T Cell Recognition.

Almost all of the sequence diversity in the TCR δ-chain is concentrated in the V-J junctional region, which led Dr. Davis and his colleagues to examine more closely the issue of TCR diversity versus immunoglobulins. Both TCR heterodimers (α:β or γ:δ) have significantly (10^4–10^7 ×) greater diversity in their junctional residues than do immunoglobulin H:L combinations and quite a bit less (10^2–10^3 ×) diversity in their V:V combinations. This difference may be explained by the fact that immunoglobulins are binding to single (and often quite large) antigenic entities, whereas TCRs are recognizing small fragments of antigens embedded in (larger) MHC molecules. Thus it seems likely that the junctional residues of TCR V regions are primarily interacting with antigens, whereas the remainder of the V region heterodimer is interacting with MHC determinants. Modeling of TCR V regions based on antibody structures and the recent solution of an MHC class I structure tend to support this. Tests of this hypothesis involving hypervariable region transfer mutagenesis and ablation experiments are under way.

II. Expression of TCRs and MHC Molecules in a Solubilizable Form.

To study T cell recognition in a cell-free system, Dr. Davis and his colleagues have developed a method for expressing TCR heterodimers in a form easily solubilizable. This involves replacing the normal transmembrane and cytoplasmic portions of TCR polypeptides with signal sequences for lipid-linked surface expression, derived from either decay-accelerating factor (DAF) or human alkaline phosphatase (HPAP). In at least one case, the resulting chimeric proteins form normal-appearing disulfide-linked α:β heterodimers. V_α and V_β epitopes present on the original TCR are preserved in the lipid-linked form, as are their approximate spatial relationships. Molecules expressed in this way are easily cleavable with the enzyme phosphatidylinositol-specific phospholipase C and isolated from immunoaffinity columns. Recently, Dr. Davis used the HPAP signal sequences to express the mouse class II MHC molecule, I-E^k, the MHC ligand for one TCR (2B4) that has been expressed in this fashion. Because the 2B4 T cell is specific for a fragment of pigeon cytochrome *c* complexed with I-E^k, the key elements of this event may soon be able to be reconstituted *in vitro*. Quantities are also sufficient to produce enough material for structural studies. Thus Dr. Davis and his colleagues hope to move the analyses of T cell recognition from a cellular readout, of necessity indirect, to direct biochemical and molecular assays.

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III. Selection of TCRs *In Vitro*.

Classical experiments in cellular immunology have long suggested that the thymus exerts considerable control over the specificity of T cells that are permitted to leave. In particular, it is believed that self-reactive T cells are eliminated (negative selection) and that only those T cells that are somehow compatible with one or the other self-MHC molecules are permitted to leave (positive selection). A very promising route toward examining these phenomena is the use of transgenic mice bearing TCR genes of known TCR specificity. These animals have large numbers of T cells bearing the appropriate receptor, and thus the selective forces operating on those receptors are much easier to study. Lines of mice have been established that express two different TCR heterodimers, both of which should be subject to positive selection based on the I-E molecule and one of which should be negatively selected in $I\text{-}A^s$–bearing mice. Results indicate that these phenomena are occurring and that expression of the original restricting element (I-E) on thymic epithelial cells is all that is necessary for positive selection to occur.

IV. The XLR Locus and a New Methodology for Subtractive cDNA Cloning.

Another area of interest involves the isolation and characterization of genes that might control differentiation in lymphocytes. One such gene is the XLR gene, which encodes a small (25 kDa) previously unidentified nuclear protein, which is weakly homologous (~15%) to intermediate filament proteins and is specifically turned on in late-stage B cells and medium-to-later stage T cells. The XLR protein is stabilized in the nucleus by zinc ions. This phenomenon has not been described previously and seems characteristic of a number of other nuclear proteins as well. The XLR protein does not have a zinc-finger motif but may bind zinc directly in some other fashion. Alternatively, there may be some structure in the nucleus that is stabilized by zinc, which in turn binds XLR. In either case, the possibility exists that intracellular free zinc concentrations may regulate the nuclear localization of a novel class of polypeptides.

A major effort is also under way to isolate other potential regulatory genes, using a subtractive hybridization and cloning scheme involving the vector λZAP. This vector has the ability to express a cDNA insert as a single-stranded plasmid circle and, by selecting such circles directly by hybridization with the relevant RNAs and hydroxyapatite chromatography, species of interest can be enriched and characterized much more quickly than was previously possible. Dr. Davis and his colleagues are particularly interested in genes that are turned on late in B cell differentiation and also in those specific for early thymocytes. Nuclear localizing proteins in either category may provide important clues about the regulation of differentiation in these cells, clues that may be applicable to cellular differentiation in general.

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GENETICS, STRUCTURE, AND FUNCTION OF HISTOCOMPATIBILITY ANTIGENS

KIRSTEN FISCHER-LINDAHL, PH.D., *Investigator*

Histocompatibility (H) antigens are traditionally divided into major and minor antigens, depending on the strength of the immune response they provoke. The major H antigens are encoded in a single gene complex, the major histocompatibility complex (MHC), whereas minor H antigens are encoded by every chromosome. Work in Dr. Fischer-Lindahl's laboratory on an unusual H antigen, maternally transmitted antigen (Mta), has led to a unified model of H antigens: they are all variations on the theme of an MHC class I or class II protein presenting a peptidic ligand. An allelic difference in the MHC protein can be a major H antigen, whereas an allelic difference in one of the peptidic ligands is a minor H antigen.

I. Mitochondrial Minor H Antigen.

A. *Processing of maternally transmitted factor (MTF).* Mta is a complex of MTF, an MHC class I molecule (Hmt), and β_2-microglobulin. Last year the 5′ end of the mitochondrial *ND1* gene was identified as the *Mtf* gene. A synthetic peptide corresponding to the first 17 amino acids of ND1 can be added to cells and will be recognized by cytotoxic T lymphocytes as if it were the mitochondrial gene product. The antigenic difference is due to allelic variation in the sixth residue, which can be isoleucine, alanine, valine, or threonine. A maximal response is obtained with the peptide at a concentration of 100 nM or more, which is very similar to observations made with viral peptide antigens. Cells must be incubated with the peptide for at least 5 h for optimal presentation of the antigen. The uptake and presentation of the peptide can be accelerated by inducing pinocytosis. All suitable target cells are capable of presenting the peptide, suggesting that no specialized antigen-processing mechanism is required. Cells treated with the ND1 peptide compete fully with target cells with the natural mitochondrial antigen, showing that only 1 of the 15–40 allelic amino acid differences in mitochondrial proteins is recognized as a minor H antigen in the Mta system.

B. *Absence of the thymus leukemia antigen (TL) on mitochondria.* The mitochondrial peptide MTF is presented by Hmt, which is encoded by a gene closely linked to *Tla*, another MHC class I gene encoding the thymocyte cell surface antigen TL. With gold-tagged monoclonal antibodies and frozen sections of intact cells, reports of association of TL with isolated mitochondria were reexamined and discarded as based on contamination of the mitochondria with plasma membrane during the isolation. In the sections of intact cells, the plasma membrane was well labeled with anti-TL and the mitochondria with a specific marker, but no specific labeling of the mitochondria with anti-TL could be detected. The techniques that were developed for fixation of mitochondria with minimal damage of epitopes will be used to study the cellular distribution of MTF.

II. MHC Class I Genes in the *Hmt* Region.

A. t *haplotypes.* The *Hmt* region on chromosome 17 is limited by two recombinational breakpoints, R4-e and R4-l, thought to be <0.7 cM apart. Two other breakpoints have been useful in the subdivision of the *Hmt* region: the end of the distal *t* inversion and the t^{w18} duplication. In recombinants between two *t* haplotypes that differ for *H-2*, *Hmt*, and *Tpx-1*, *Hmt* segregates with *H-2* rather than with *Tpx-1*, showing that it is included in the *t* inversion. Because the currently known MHC class I genes of the *Hmt* region all have characteristic, conserved restriction fragment length polymorphisms (RFLP) in *t* haplotypes, they must lie within the *t* inversion and therefore belong to the proximal end of the *Hmt* region, closest to *Tla*.

B. *Cloning new genes.* The *R4-l* haplotype carries the *Hmt* region from *Mus musculus castaneus* (Hmt^b) on a chromosome 17 otherwise derived from the laboratory strains C3H and C3H.SW (Hmt^a). Because of the genetic divergence of the mice involved, this haplotype is well suited for RFLP analysis. Digests of genomic DNA from the recombinant and parental strains were screened with a probe for the conserved exon 4 of MHC class I genes, and several fragments that must map to the *Hmt* region were identified and cloned from band libraries. The same panel of strains has also been used to map two previously cloned MHC class I genes, *Thy19.4* and *Mb1*, to the *Hmt* region.

The extremely low level of expression of *Thy19.4*, except in the thymus, and negative results from transfection experiments suggest that it is not *Hmt*. Similarly, the lack of evidence for expression of *Mb1*

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rules out that it is *Hmt*. The *Mb1* probe cross-hybridizes with a second fragment that also was mapped to the *Hmt* region; this could represent a sixth class I gene in that region.

C3R1 was cloned as a 5 kb *Eco*RI fragment, containing the 3′ end of a class I gene. With a probe derived from this clone, two cosmids with the complete gene have been isolated, one of which contains an additional, intact class I gene, *CRW2*. Since *C3R1* has a stop codon in the third exon, and no mRNA was detected with an exon 3 probe, it is a poor candidate for *Hmt*. *CRW2* is interesting in that exons 2 and 3 differ strikingly from all other class I genes (although the glycosylation site in the α1 and the two cysteines in the α2 domain are conserved), whereas exon 4 shows a normal level of similarity and the gene encodes a proper transmembrane region and a short cytoplasmic tail. *CRW2* appears to be an intact class I gene, but it is not yet known whether it is expressed.

C. *A candidate for Hmt. R4B2* was first cloned from Hmt^b DNA as a 17 kb *Bgl*II fragment. The homologue from Hmt^a mice has since been isolated in the form of one cosmid and several cDNA clones. The amino acid sequence of *R4B2* shows about 15%, 65%, 55%, 80%, and 30% similarity to known class I genes for exons 1, 2, 3, 4, and 5, respectively. It has a single glycosylation site at residue 86 in the α1 domain, a hydrophobic transmembrane domain, and a cytoplasmic tail of eight amino acids. *R4B2* mRNA is easily detectable in thymus, spleen, lymph nodes, liver, and kidney.

The properties of *R4B2* are consistent with what is known about *Hmt*. *R4B2* shares a number of conserved, structurally important residues and has sufficient sequence similarity to other MHC class I genes to suggest that it would fold in the same manner. No charged residues point directly into the peptide-binding site modeled after the HLA-A2 structure.

III. T Cell Receptor for Mta.

Recent reports have suggested that γ/δ T cell receptors (TCR) recognize MHC molecules encoded in the distal end of the complex. Rabbit anti-TCRα and anti-TCRγ sera were therefore used to immunoprecipitate the surface-labeled TCR of a number of cytotoxic T cell lines specific for Mta. All cells gave a strong band with anti-TCRα and none with anti-TCRγ, showing that they all use α/β-receptors.

IV. New $H\text{-}2K^b$ Mutant.

Not all changes in MHC molecules result in strong T cell immune responses. A spontaneous mutant was identified among the progeny of a backcross of *R4-1* to C57BL/6J, because it had lost the epitope of the $H\text{-}2K^b$ molecule recognized by monoclonal antibody B8-24-3. Whereas the mutation has affected the binding of several monoclonal antibodies, whose epitopes have been mapped to the loop area of transition from α-helix to β-sheet around residues 77–90 of the α1 domain, the mutation does not affect T cell recognition of the $H\text{-}2K^b$ molecule. The mutant does not reject skin from the parental strain, and there is no T cell proliferation or killing induced *in vitro* between mutant and parent. In a collaboration with the laboratory of Dr. Larry Peace (Mayo Clinic), the mutant gene was sequenced and the mutation identified as a lysine to alanine transition in residue 89.

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MOLECULAR CHARACTERISTICS OF THE EFFECTOR PATHWAYS OF IMMUNOLOGICAL RESPONSES

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Dr. Goetzl's studies of the mediators of effector pathways of the immune system this year have identified high-affinity receptors specific for leukotrienes and platelet-activating factor (PAF) on human neural cells, revealed the genetic message encoding human lymphocyte receptors for the neuropeptide vasoactive intestinal peptide (VIP), and defined the range of functions of the human monokine designated fibroblast-activating factor (FAF) and the expression of FAF in fibrosing diseases.

I. Neural Receptors for Leukotrienes and PAF.

Dr. Goetzl and his colleagues have shown recently that central nervous system tissue and cultures of isolated human neural cells generate both leukotrienes and PAF. Physiological stimuli and trauma increased the levels of neural generation of the lipid mediators, and specific antagonists of some of the mediators were shown by Dr. F. H. Valone and Dr. Goetzl to reduce the neurological damage after trauma. That the effects of locally generated lipid mediators on several neural functions are dependent on their recognition by stereospecific receptors was supported by the results of direct binding studies. Dr. Catherine Koo and Dr. Goetzl found that the U-373 line of human glioblastoma/astrocytoma cells bound [^{3}H]LTB_4 (leukotriene B_4) and [^{3}H]LTC_4 specifically, with respective K_d values of 20–50 and 60–80 nM. Similarly, they observed that NBII human neuroblastoma cells bound [^{3}H]LTB_4 and [^{3}H]LTC_4 with respective K_d values of 30–60 and 20–40 nM. The receptors for leukotrienes on both types of neural cells resembled the low-affinity subsets of the corresponding receptors of human neutrophils and exhibited the same specificity.

Neural receptors for PAF are coupled principally to cellular growth and differentiation and, in contrast to the leukotriene receptors, differed in affinity and specificity from monocyte and platelet receptors for PAF. Drs. Valone and Goetzl found that NGP neuroblastoma cells bear a mean of 850 receptors for PAF, with a mean K_d of 1.2 pM, that recognize 2-*O*-methoxy-PAF with far higher affinity than monocyte receptors. The capacity of both 2-*O*-methoxy-PAF and PAF to stimulate NGP cell proliferation and neurite outgrowth confirmed the unique specificity of the neural receptors for PAF.

II. Mediation of Immunity by Somatostatin, VIP, and Mast Cell–Derived Variants of VIP.

The capacity of somatostin (SOM) and VIP to suppress T cell proliferative and synthetic activities and to inhibit selectively the synthesis of IgA and IgM by mixed lymphocytes is a function of specific recognition of the neuropeptides by separate subsets of T and B cell receptors. Dr. Goetzl and Dr. Sunil P. Sreedharan focused recently on two lines of human cultured lymphocytes that bind SOM and VIP: the Jurkat leukemic T cell and the U-266 myeloma cell. They found that Jurkat T cells express two sets of a mean of 144 and >100,000 SOM receptors, with respective K_d values of 3 pM and 66 nM, and one class of a mean of 12×10^3 VIP receptors with a K_d of 5.2 nM. U-266 cells similarly bear two sets of a mean of 1,295 and >100,000 SOM receptors, with respective K_d values of 5 pM and 100 nM, and one class of 41×10^3 VIP receptors with a K_d of 7.6 nM. The specificity of the lymphocyte receptors for VIP, determined by Drs. Goetzl and Sreedharan from the rank order of affinity of a series of substituents and analogues of VIP, differed from that of neural and endocrine cell receptors. Expression-cloning techniques are being used to investigate the genetic determinants of the Jurkat and U-266 cell receptors for VIP, in order to elucidate the structural bases for their distinctive specificity.

U-266 and Jurkat cell cDNA libraries were size-selected, inserted into the CDM8 expression vector, transfected into COS cells, and enriched for VIP receptor message by flow cytometric selection, using an antibody to the 47 kDa affinity-crosslinked VIP receptor protein of Molt-4b human T lymphoblasts. Drs. Goetzl and Sreedharan, in collaboration with Dr. Sue O'Dorisio, then identified and isolated a positive transfectant containing a 405 bp cDNA that endowed COS cells with both specific binding of the anti-VIP receptor antibody and high-affinity binding of [^{125}I]VIP. Several 800–1,500 bp cDNAs that comprise the full-length cDNA for the VIP receptor have been recovered from the U-266 library, by employing the 405 bp cDNA as a (^{32}P) probe for hybridization analyses of *Escherichia coli* transformants.

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III. Human Monocyte-derived FAF in Wound Healing and Fibrosis.

The range of effects of highly purified native FAF on human dermal fibroblasts has been delineated by Dr. Goetzl and Dr. Christoph W. Turck in a variety of assay systems, in comparison with other growth factors. At the different respective concentrations that elicited the same levels of fibroblast proliferation, transforming growth factor-β (TGF-β), interleukin-1 (IL-1), acidic fibroblast growth factor (aFGF), and FAF all stimulated fibroblasts to generate similar quantities of prostaglandin E_2 and proteoglycans. The magnitude of the stimulation of proteoglycan synthesis varied most widely among the factors, and TGF-β had the least effect. In contrast, the production of collagen evoked by FAF was found to be only minimal, compared with that induced by IL-1 and aFGF. Thus the functional definition of FAF as a selective fibroblast growth stimulus, with no effect on endothelial or smooth muscle cells, has been extended to a distinctive profile of effects on fibroblasts.

Knowledge of some of the polypeptide structure of FAF has permitted studies of expression in alveolar macrophages (AMs) of patients with diverse inflammatory and fibrosing diseases of the lung. AM-derived cDNAs encoding messages for FAF and 10 other growth factors were amplified by polymerase chain reaction (PCR) with a pair of 3′ and 5′ primers for each factor. Whereas AMs from three subjects with no lung disease expressed messages for IL-1β, TGF-β, and occasionally insulin-like growth factor 1 (IGF-1), these and epidermal growth factor (EGF), TGF-α, IL-1α, and/or platelet-derived growth factor (PDGF) were found in three patients with inflammatory and three patients with fibrosing lung diseases. Analyses of genetic messages in immune effector cells thus may provide useful prognostic information in some multigenic diseases.

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NORMAL HUMAN T CELL GROWTH AND PATHOGENIC HUMAN RETROVIRUSES

WARNER C. GREENE, M.D., PH.D., *Investigator*

Investigations in Dr. Greene's laboratory have focused on four principal areas: 1) the structure and function of the human receptor for interleukin-2 (IL-2, T cell growth factor), 2) transcriptional regulation of the IL-2 receptor-α (IL-2Rα) gene, 3) function and mechanism of action of the *tax* and *rex* trans-regulatory genes of the type I human T cell leukemia virus (HTLV-I), and 4) regulation of type 1 human immunodeficiency virus (HIV-1) gene expression by various viral and cellular factors.

I. Human High-Affinity IL-2 Receptor.

The functional high-affinity IL-2 receptor is now known to comprise at least two different IL-2–binding subunits, IL-2Rα (p55, Tac) and IL-2Rβ (p70/75). Coexpression of cDNAs for these two subunits leads to functional receptor display in lymphoid, but not in nonlymphoid, cells. These findings strongly suggest that yet additional structural subunits are involved in high-affinity receptor assembly. Dr. Greene and his colleagues have recently prepared monoclonal antibodies that appear to react with a third component of this receptor complex, a 52 kDa IL-2Rγ chain. IL-2Rγ is expressed in a broader array of cells than either IL-2Rα or IL-2Rβ. Immunoprecipitates formed with the anti-IL-2Rγ antibodies contain high levels of tyrosine kinase activity. Although it remains unknown whether IL-2Rγ is the receptor-associated tyrosine kinase, recent studies indicate that this second messenger system is intimately involved in IL-2–induced growth signal transduction.

II. Transcriptional Regulation of the IL-2Rα Gene.

The prior studies of Dr. Greene and his colleagues have shown that a κB enhancer element present in the 5′-flanking region of the IL-2Rα gene contributes significantly to the inducible nature of IL-2Rα gene expression. Mitogens [phytohemagglutinin (PHA), phorbol 12-myristate 13-acetate (PMA)], cytokines [tumor necrosis factor-α (TNF-α)], and the Tax protein of HTLV-I all induce the expression of κB-specific proteins that interact with this IL-2Rα enhancer element. At least four polypeptides of different sizes react with the IL-2Rα κB element, as assessed in DNA-protein crosslinking assays. A related κB enhancer is also present in the IL-2 gene that reacts with these same inducible trans-acting factors, albeit with lower affinity. The common action of these κB-specific factors is likely involved in the coordinate expression in T cells of this growth factor and growth factor receptor gene. Sequences positioned immediately upstream and downstream of the IL-2Rα κB enhancer also contribute to the overall regulation of IL-2Rα gene expression. Specifically, a 5′-binding site for a 56 kDa constitutively expressed protein is required for PMA, but not Tax, induction of this transcription unit. In contrast, a CArG box and Sp1 site located immediately 3′ of the κB element are needed for both PMA and Tax activation. Finally, an upstream negative regulatory element (NRE) has also been identified in IL-2Rα promoter that attenuates both basal and induced responses. This NRE specifically interacts with constitutively expressed 50 kDa protein. This same 50 kDa factor binds to an NRE present in the HIV-1 long terminal repeat (LTR). Thus the IL-2Rα promoter and HIV-1 LTR are not only coregulated by the same set of positive host factors (e.g., NF-κB) but also may be subject to negative regulation by the same inhibitory cellular protein.

III. HTLV-I Trans-Regulation: Action of the Tax and Rex Proteins.

The pathogenic HTLV-I retrovirus has been etiologically linked with the aggressive and often fatal adult T cell leukemia and with the progressive neurological syndrome tropical spastic paraparesis. HTLV-I encodes two trans-regulatory proteins, Tax and Rex, each of which is required for viral replication. The 40 kDa Tax protein serves as a powerful trans-activator of the HTLV-I LTR and various cellular genes involved in T cell growth. These cellular targets include the genes encoding IL-2 and IL-2Rα. Tax does not directly bind to DNA but indirectly activates these cellular genes, in part by inducing the expression of the κB-specific family of proteins. In contrast, Tax activation of the HTLV-I LTR involves yet a second set of cellular proteins, indicating that this viral trans-activator interfaces with multiple host transcription factor pathways.

The HTLV-I Rex protein functions post-transcriptionally to regulate viral structural gene expression. Rex acts in part by inducing the nuclear export of the unspliced or singly spliced viral mRNAs that encode the structural proteins (Gag and Env) required for the assembly of infectious virions. In the absence of Rex these incompletely processed

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viral transcripts are normally excluded from the cytoplasm. Rex functions in a sequence-specific manner requiring the presence of a Rex response element located in the 3′ LTR. This Rex response element appears to correspond to a complex RNA stem-loop structure (279 nucleotides) that can function at any position within the viral RNA but must be present in the sense orientation. Recent studies have revealed that the Rex protein is able to replace the biological function of the Rev protein in the HIV-1 system, functioning through a related Rev response element. However, this rather remarkable genetic complementation is nonreciprocal, as the HIV-1 Rev protein is unable to function via the HTLV-I Rex response element. Mutational analysis of the HTLV-I Rex protein has also led to the delineation of two peptide domains specifically involved in nuclear targeting of this trans-activator and its effector function. Mutations in this latter effector domain yield trans-dominant repressor proteins that exert inhibitory effects on the growth of both HTLV-I and HIV-1. These trans-dominant Rex mutants thus join a small but expanding group of dominant negative viral proteins that hold promise as a novel approach to antiviral therapy.

IV. Regulation of HIV-1 Replication.

Prior studies have suggested that the 27 kDa Nef protein of HIV-1 functions as a negative factor by downregulating HIV-1 LTR-mediated transcription. To assess the possible role of Nef in the initiation or maintenance of latent forms of HIV-1, Dr. Greene and his colleagues analyzed the biological effects of Nef, using several *nef* expression vectors for transfection of multiple cell types. Although these expression plasmids directed the synthesis of the expected cytoplasmic and myristylated 27 kDa protein, Nef exhibited no detectable inhibitory effects on the HIV-1 LTR in either lymphoid or macrophage cell lines or primary T cells. Furthermore, no differences in the replication of nef^+ and nef^- HIV-1 proviruses were discerned. Thus, under the conditions of these assays, Nef does not function as a negative factor. The true action of this regulatory viral polypeptide, which appears conserved in HIV-1, HIV-2, and simian immunodeficiency virus type 1 (SIV-1), remains elusive.

Dr. Greene is also Professor of Medicine at the Duke University Medical Center.

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DEVELOPMENTAL CONTROL OF GENE EXPRESSION

RUDOLF GROSSCHEDL, PH.D., *Assistant Investigator*

The research in Dr. Grosschedl's laboratory is focused on the molecular mechanisms that underlie the developmental regulation of immunoglobulin (Ig) gene expression. Ig μ heavy-chain and κ light-chain genes are expressed only in B lymphocytes. Transcripts from these genes are generated in a temporally ordered manner during B cell differentiation, with the appearance of μ transcripts preceding the synthesis of κ mRNA.

I. Tissue-specific and Temporal Regulation of Immunoglobulin Gene Expression in Transgenic Mice.

To examine the function of known regulatory sequence elements and individual factor-binding sites for the developmental expression pattern of Ig genes *in vivo* and to identify new sequence elements, Dr. Grosschedl and his colleagues introduced wild-type and mutated μ genes into the mouse germline. Deletion of the enhancer abrogated expression of the exogenous μ Ig gene at any stage of the B cell lineage. The Ig heavy-chain (IgH) enhancer interacts with many nuclear factors. Although most of the Ig enhancer-binding factors are present in all tested cell types, one of these factors, Oct-2, is found only in B cells. To examine the role of Oct-2 in the establishment of the correct developmental expression pattern *in vivo*, the laboratory introduced μ genes carrying point mutations in the Oct-2–binding sites into the mouse germline. Mutations in the Oct-2–binding sites of the promoter and enhancer decreased the level of μ gene expression in B cells by two orders of magnitude. The mutations in the Oct-2–binding site, however, did not abrogate the cell-type-specificity of μ gene expression. These data suggest that Oct-2 is an important, but not the only, determinant for the lymphoid-specific expression of the μ gene. The occupancy of the wild-type and mutated enhancer was analyzed by *in vivo* genomic footprinting to examine whether the point mutations in the Oct-2–binding site of the enhancer abrogate binding of any of the other factors. In the mutated enhancer of the transgene, another factor-binding site that, by itself, is not affected by the mutation was also found to be unoccupied, indicating that the binding of a factor to this specific site requires an interaction with Oct-2.

To determine the molecular basis for the sequential expression of Ig heavy- and light-chain genes during B cell differentiation, Dr. Grosschedl and his colleagues constructed hybrid Ig genes in which individual regulatory sequences of either μ heavy- or κ light-chain genes were interchanged. After gene transfer into the mouse germline, the temporal expression pattern of the transgenes was examined. Expression of the hybrid transgene in which the μ heavy-chain enhancer was replaced with the κ light-chain enhancer resulted in a change in the temporal expression pattern. During mouse development, expression of the hybrid transgene was delayed by at least one day gestation. Moreover, in pre–B cells representing the early stage of the B cell lineage, expression of the hybrid transgene was low when compared with the intact μ gene. In contrast, in more mature B cells the level of expression of the hybrid gene was similar to that of the μ gene. Thus the differential activity of the κ enhancer in pre–B and mature B cells appears to determine, at least in part, the sequential expression of Ig genes *in vivo*.

II. Role of Enhancer Sequences in Maintenance of the Active Transcriptional State During Cell Proliferation.

The pattern of gene expression of mammalian cells can be stably propagated from mother to daughter cells. The maintenance of the transcriptional state has been attributed to the stable propagation of transcription complexes and to stable modifications of DNA or chromatin. If transcriptional complexes persist during DNA replication, and if modifications of DNA or chromatin are inheritable, the transcriptional state of a gene could be propagated in the absence of the genetic elements required for establishment of its expression pattern. In particular, enhancer elements have been considered only transiently required to establish the assembly of a transcription complex.

An experiment was devised to test whether the enhancer can confer upon a gene "memory" of its active transcriptional state. Pre–B cell lines were generated carrying a transfected μ gene inserted stably at various chromosomal locations. The transfected μ gene construct contained an Ig enhancer positioned between D and J recombination signals. These signals are recognized by a site-specific recombinase in pre–B cells and are joined together, resulting in a deletion of internal sequences. Because the deletion of the enhancer in the trans-

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fected μ gene construct could be controlled, the cells were allowed to establish μ gene transcription in the presence of the enhancer and subsequently a portion of the transfected cells was allowed to delete the enhancer from the μ gene by site-specific D-to-J rearrangement. This experiment showed that deletion of the enhancer from an actively transcribed gene reproducibly resulted in switching off of μ gene expression. Thus the enhancer seems to be required for both establishment and maintenance of μ gene expression.

III. Isolation and Characterization of Lymphocyte-specific cDNA Clones.

To identify a set of new markers for individual stages of the lymphocyte cell lineage and to find gene products that are important for the lymphoid cell differentiation pathway, Dr. Grosschedl and his colleagues isolated novel lymphocyte-specific cDNA clones. By subtractive hybridization of a pre–B cell cDNA library with cDNA from an erythroid cell line, cDNAs from genes that are expressed only in cells of the B and T lymphocyte lineage were isolated. These genes show three distinct expression patterns. Some are expressed in cell lines representing T cells and early stage pre–B cells but are not expressed in cells of the mature B cell stage. Another set of genes is expressed only in B lymphocytes, whereas a third set is expressed in all analyzed B and T cell lines. Analysis of the 5′-terminal cDNA sequences indicated that five of the isolated clones represent previously unknown genes. The nucleotide sequences of these cDNA clones are being determined, and the cDNAs are being expressed in bacteria to obtain protein for the generation of antibody.

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

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MOLECULAR MECHANISMS OF CYTOKINE IMMUNOREGULATION

KENNETH J. HARDY, M.D., PH.D., *Assistant Investigator*

The cytokine network directly or indirectly modulates, among other processes, inflammation, hematopoiesis, bone and muscle growth, wound healing, scar formation, tumorigenesis, and immune responsiveness. Interferon-γ (IFN-γ) occupies a central role in that network as a potent modulator of both the affector and the effector limbs of the immune response. Although promising as a therapeutic agent for immunodeficiency states, infectious and neoplastic diseases, and autoimmunity, IFN-γ has perhaps more often been evoked in the pathogenesis of such disorders. Aberrant expression of this cytokine has been implicated in acquired immune deficiency syndrome (AIDS), multiple sclerosis, chronic granulomatous disease of childhood, and several inflammatory arthritides. This clinically relevant molecule is broadly interactive with other members of the cytokine system and, as demonstrated in Dr. Hardy's laboratory, can be influenced both positively and negatively by a variety of stimuli, including other cytokines, as well as its own gene product. To facilitate an understanding of genetic regulation within the cytokine network as a whole, using the IFN-γ gene as a model, this laboratory has identified human cellular systems, both cultured and primary, in which this gene can be interactively and differentially modulated. These cells are then examined with regard to cell surface triggers, proximal metabolic pathways, and the molecular structure and function of their IFN-γ genes.

I. Definition of Cellular Systems Differentially Regulating IFN-γ Gene Expression.

Several human T cell tumor lines (including Jurkat, Hut 78, and D4) as well as non–T cell lines (such as Raji and HeLa) have been characterized on the basis of their tissue-specific and/or differential expression of human IFN-γ. More recently, Dr. Hardy's laboratory has begun isolating lymphocytes directly from normal human peripheral blood and fractionating them into total peripheral blood lymphocytes (PBLs); erythrocyte rosette positive and negative; and $CD4^+$, $CD8^+$, $CD45R^+$, $CD29^+$, $CD16^+$, $CD19^+$, and $CD15^+$ cells. Such mononuclear cell subsets have been analyzed for their regulated expression of IFN-γ, interleukin-2 (IL-2), and other cytokines. Titratable modulation of IFN-γ gene expression by various combinations of antigen-presenting cells (APCs), lectin–phorbol ester combinations, and/or cytokine-soluble factors, has provided insights into cytokine regulation impossible to obtain using tumor cell lines. Primary lymphoid cells and their subsets, when used in transient transfection studies, have become an important and physiologically relevant system for the molecular genetic analyses of lymphokine gene regulation.

II. Surface Triggers and Proximal Metabolic Pathways Leading to T Cell Activation and Lymphokine Gene Expression.

The metabolism of the major T cell membrane phospholipid, phosphatidylcholine (PC), has been investigated in Dr. Hardy's laboratory and demonstrated to be involved in T cell signal transduction pathways generally attributed to phorbol 12-myristate 13-acetate (PMA) (and/or APCs). Specific synthesis (turnover) of phosphatidylinositol (PI) and PC in Jurkat, total PBLs, and their subsets has been found to depend on stimulation with either phytohemagglutinin (PHA), PMA, or both. Under conditions of T cell activation and IFN-γ gene induction, turnover of both PC and PI are increased significantly. Neither neonatal cord blood lymphocytes nor "naive" ($CD45R^+$) lymphocytes expressed IFN-γ upon T cell activation, but both demonstrated normal PI and PC turnover patterns, suggesting that differential inactivity of the human IFN-γ gene occurs through mechanisms distal to these metabolic pathways. In a perhaps related observation, no effects of APCs, IL-1, and leukotrienes (normal upregulatory signals to IFN-γ expression) on PI or PC pathways were seen.

III. Structural and Functional Analysis of IFN-γ Gene Regulation.

A. *Positive effectors of IFN-γ gene regulation.* Several physiologic agents have been implicated as upregulators of IFN-γ gene expression. Definitive evidence has now been obtained from Dr. Hardy's laboratory that IFN-γ can autosuperinduce its own gene expression in a dose-dependent fashion. Both higher steady-state levels of IFN-γ transcripts and enhanced secretion of its biologically active product were observed. Highly purified $CD3^+$ cells, either alone or reconstituted to autologous monocytes, were poorly responsive in this regard, arguing against an autocrine mechanism. This was in sharp contrast to the strong upregulatory response of these same cells to accessory stimulation with another cytokine, IL-2. Preliminary data point to an important role for cells

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in the nonrosetteable fraction in regulating the extent and duration of the IFN-γ autosuperinduction response. The implications of these phenomena in autoimmune dysfunction are under investigation.

B. *Negative effectors of IFN-γ gene expression.* A crude, murine-derived T cell suppressor factor (TSF) was shown to contain a strong downregulator of the IFN-γ gene expression, while having no effect on IL-2, IL-2 receptor (IL-2R), or actin expression. TSF was subsequently found to contain measurable levels of transforming growth factor-β (TGF-β), a factor recently reported to be similarly capable of suppressing biological expression of several human cytokines. Dr. Hardy's laboratory subsequently examined the effect of TGF-β on IFN-γ gene expression by activated human PBLs, clearly demonstrating a powerful downregulatory effect on IFN-γ gene expression. Recent studies have implicated the monocytes as the key cellular element facilitating this downregulatory effect.

C. *Structural and functional analyses of the IFN-γ gene.* Previous structural studies from Dr. Hardy's laboratory had identified three regions of the human IFN-γ gene, two upstream and one intronically located, and implicated them functionally, via interdomain communication, in that gene's positive and negative regulation. By constructing and transfecting a number of reporter plasmid constructs into human and murine cell lines, Dr. Hardy and his colleagues have functionally identified several cis-acting regulatory regions in the human IFN-γ gene. A tissue-nonspecific enhancer element has been localized to a 230 bp fragment in the first intron of the human IFN-γ gene. Tissue-nonspecific DNase I footprints have also been identified in this same fragment. It was demonstrated that the upstream 2.3 kb of the IFN-γ gene, while inactive in fibroblasts, can direct activation-specific upregulation of reporter constructs transiently transfected into murine T cells. Deletion analyses localized the sequences involved in the induction process to the proximal 5′ 550 bp. Dr. Hardy's laboratory optimized electroporation conditions to allow transfection of expression constructs directly into fully differentiated, primary human lymphocytes (and/or their isolated subfractions). By this approach, several cis-acting genetic elements were identified in this proximal 5′ region. A T cell–specific, constitutive enhancer was localized to the most proximal 215 bp. Furthermore, a T cell–specific DNase I footprint was identified at position −172 to −156. Deletion analyses suggested dominant suppression of this enhancer region by more upstream negative regulatory elements. The first functional confirmation of a T cell–specific, negative regulatory element was made in this laboratory, and its orientation dependency was demonstrated. These data support a model of cytokine gene induction that involves T cell–specific derepression of negative elements otherwise dominantly suppressing a potent, T cell–specific, constitutive enhancer. Clonal identification of T cell–specific trans-acting factors is under way.

Dr. Hardy is also Assistant Professor of Medicine, Microbiology and Immunology, and Cell Biology, and Attending Physician at Baylor College of Medicine and affiliated hospitals.

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STRUCTURE AND FUNCTION OF COMPLEMENT RECEPTORS

V. MICHAEL HOLERS, M.D., *Assistant Investigator*

One of the many functions of the complement system is to place activated forms of complement components C3 and C4 onto the surface of targets. These targets include immune complexes, as well as foreign soluble and particulate antigens. After covalent attachment of activated C3 on the target, specific proteolytic processing steps occur on the C3 molecule that result in at least three major ligand forms. These forms each have a high affinity for a distinct C3 receptor. Depending on the cell type in which these receptors are expressed, C3-containing ligands are either transported to other tissue sites or are bound, internalized by phagocytic and/or endocytic mechanisms, and then processed. The intracellular fate of specific ligand-receptor complexes is incompletely understood.

Dr. Holers and his colleagues have been analyzing one of the complement receptors, complement receptor 2 (CR2), and have utilized it as a model for the function, regulation of expression, and mechanisms of action of these receptors. CR2 is an ~145 kDa single-chain receptor expressed primarily on human B cells, follicular dendritic cells, and some epithelial cells. It is a member of a family of complement regulatory and binding proteins, known as the regulators of complement activation, which is found in a single genetic locus on human chromosome 1q32. CR2 interacts with a peptide sequence on a form of activated and proteolytically processed C3 known as C3d. In addition, recent work has established that CR2 also functions as the receptor for the Epstein-Barr virus (EBV). EBV is a human pathogen that causes most clinical cases of infectious mononucleosis and is also causally associated with lymphoid and nonlymphoid tumors.

CR2 is present primarily on mature, surface IgM-positive B cells and is not found at detectable levels on early pre–B cells, lymphoid progenitors, or late fully differentiated immunoglobulin-secreting B cells. Binding of ligand or some monoclonal antibodies to CR2 results in a coproliferative stimulus to resting peripheral or tonsillar B cells. CR2 is part of a growing family of B cell membrane proteins that are developmentally regulated and are probably involved in the control of steps in B cell ontogeny and activation.

Another receptor expressed on B cells in a developmentally regulated fashion is the human fibronectin receptor VLA-5. VLA-5 is a member of the integrin gene family. Members of this family function in adherence of cells to matrix proteins and to other cells. In a number of model systems, these receptors play important roles in targeting of cells to specific sites during development and to other sites later in life.

Dr. Holers and his colleagues have been studying the expression of VLA-5 on phagocytic cells, because it appears to help regulate the function of complement receptors on these cells. The laboratory has also recently begun to analyze the function and regulation of expression of this receptor on human B cells.

I. Complement Receptor 2.

Previous studies by Dr. Holers and others had indicated that the primary structure of CR2 consists of either 15 or 16 short consensus repeats (SCRs), followed by transmembrane and intracytoplasmic domains. SCRs are ~60–70 amino acids in length, have four invariant cysteines and a tryptophan at the same position in each repeat, and have other highly conserved but not invariant amino acids at other positions. This type of repeat is found in complement regulatory proteins and also increasingly in other proteins and receptors, such as those involved in lymphocyte homing.

One goal of the laboratory is to identify and characterize the functional domains on CR2. These include those in the extracellular domain involving the ligand-binding sites for both C3d and EBV. Recent studies using deletion mutagenesis have indicated that single binding sites are found for both of these ligands in the most amino-terminal SCRs of CR2. Previous work on the genomic organization of CR2 had shown that the extracellular domain evolved through successive duplications in the genome of a four-SCR-containing motif. Despite there being four duplications of a four-SCR-containing repeat, only one SCR appears to contain a binding site, and it is in the most amino-terminal duplication. The function of the more proximal SCRs remains unclear. They may act to extend the distal repeat away from the cell membrane, to interact with other membrane receptors or other CR2 molecules to form multireceptor complexes, or to mediate or facilitate transmembrane signaling events. Studies are ongoing to address these questions. CR2 also has a short intracellular domain. Deletion of this domain leads to defects in the ability of CR2 to me-

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diate EBV infection. Further studies are ongoing regarding the role of this domain in EBV infection and in signal transduction and receptor trafficking.

Dr. Holers and his colleagues have also placed the CR2 cDNA in a number of recombinant vector backgrounds, including retroviral-based backgrounds. These are being utilized to transfer CR2 to various cell lines and naive primary cells. The functions of CR2 will be analyzed in these different cell backgrounds. These studies will increase understanding of the host cell restriction of CR2 function in regard to EBV infection and C3d binding and processing.

A major promoter region in the CR2 gene has been identified. Because CR2 is developmentally regulated during B cell ontogeny, an extensive analysis of this region should allow further understanding of the nucleotide sequence motifs, DNA-binding proteins, and other regulatory steps of importance in expression of genes during B cell ontogeny.

To analyze further the evolution and function *in vivo* of CR2, the laboratory has recently identified and cloned mouse CR2 cDNA and genomic homologues. Ongoing analysis of these clones has indicated a high degree of sequence conservation to human CR2 in both cDNA and genomic structure. Construction of antibody and other probes to the protein predicted by this cDNA are ongoing. These studies will allow a more definitive analysis of the evolution of CR2 and the *in vivo* role of CR2 in the immune response.

II. Human Phagocytic Fibronectin Receptor (VLA-5).

Dr. Holers has recently undertaken an analysis of VLA-5 expression on human B cells. He has found VLA-5 on pre–B cells but not on mature B cells. Initial evidence indicates that activation of human B cells may result in reexpression of VLA-5. These results may have importance in cellular homing of these cells or in adherence to inflammatory sites.

The laboratory has also analyzed expression of VLA-5 on phagocytic cells, using mouse peritoneal macrophages as the primary model. The regulation of the expression of this receptor by inflammatory mediators has been demonstrated. Resting cells are VLA-5 low, and activated cells are VLA-5 high. This suggests that the regulation of surface expression of these receptors is important in either homing of cells to inflammatory loci or adherence to these sites.

Dr. Holers is also Assistant Professor of Medicine and Pathology at Washington University School of Medicine and Assistant Physician at Barnes Hospital, St. Louis.

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SELECTIVE INFLUENCES ON T CELL DEVELOPMENT

CHARLES A. JANEWAY, JR., M.D., *Investigator*

Studies in Dr. Janeway's laboratory are focused on environmental influences on the development of specificity in T lymphocytes. During the past year, progress in several areas has led to the formulation of new hypotheses about several of these environmental influences and the development of a new model of T cell development. This model seeks to account for the central mystery in this process, the generation of a repertoire of receptors that is selected for the recognition of foreign antigens presented by self major histocompatibility complex (MHC) molecules, while at the same time being unresponsive to these same MHC molecules or self peptide fragments bound to these MHC molecules. This model is based on several new findings.

I. CD4: A Physical Component of the T Cell Receptor.

The cell surface molecule CD4 is expressed on T cells that recognize self class II MHC molecules. Studies in Dr. Janeway's laboratory have shown that CD4 physically associates with the T cell receptor during recognition of antigen:class II MHC complexes and that this association potentiates signaling through the receptor by ~100-fold. Crosslinking CD4 in the context of the T cell receptor activates tyrosine kinases that phosphorylate novel cytoplasmic substrates that are now being characterized. Because CD4 interacts in a defined orientation with both class II MHC molecules and the T cell receptor, the orientation of the T cell receptor and class II MHC molecules must also be fixed. Dr. Janeway has proposed the term *co-receptor* to describe the function of CD4 and of the class I MHC–binding molecule CD8.

II. Orientation of T Cell Receptor:MHC Interaction.

Antigen-presenting cells express a class of molecules Dr. Janeway terms *co-ligands*. Dr. Janeway's laboratory has studied the *Mls* co-ligands and introduced the staphylococcal enterotoxins as models for the analysis of *Mls* co-ligands. Staphylococcal enterotoxins were shown to bind to class II MHC molecules and the Vβ segment of the T cell receptor and to stimulate T cell activation directly. Staphylococcal enterotoxins also induce clonal deletion of T cells bearing certain Vβ-encoded receptors. This assay was used to show that tolerance mediated by such substances is more sensitive than is activation, allowing a margin for safety in the clonal deletion process in the thymus. Co-ligands also can potentiate antigen presentation. Because co-ligands also bind specifically to the T cell receptor and the class II MHC molecule, they further orient the T cell receptor:ligand interaction.

III. Co-ligand–Driven Clonal Deletion and Prevention of Murine Diabetes.

Dr. Janeway's laboratory has isolated cloned T cell lines that will transfer autoimmune diabetes to syngeneic, irradiated mice. Both a CD4 and a CD8 T cell are required to transfer diabetes. Both cloned lines express T cell receptors encoded by Vβ5 genes. T cells whose receptors are encoded by Vβ5 genes are deleted in mice expressing I-E class II MHC molecules and a suitable co-ligand, and diabetes-prone mice expressing I-E do not develop disease. Moreover, these cloned lines can cause disease in I-E mice, demonstrating no protective effect of I-E on host tissues. Thus the protective effect of I-E is almost surely due to its ability to present a co-ligand for deletion of diabetes-inducing, Vβ5-bearing T cells. Finally, depletion of Vβ5-bearing T cells from T cell populations obtained from diabetic mice eliminates their ability to transfer disease to normal recipients.

IV. Aberrant Expression of Self Peptides in Thymic Cortical Epithelium.

Positive selection of the T cell repertoire occurs on thymic cortical epithelium. Dr. Janeway's laboratory has prepared a monoclonal antibody to a self peptide bound to a self class II MHC molecule. This complex is expressed by all class II MHC–expressing cells except thymic cortical epithelium. This suggests that the ligands involved in positive selection are molecularly distinct from those involved in negative selection or peripheral T cell activation. This may explain how T cells can be positively selected by self MHC and yet have specificities not found on peripheral cells that would cause clonal deletion.

V. Involvement of Crosslinking and Conformational Change of the T Cell Receptor in T Cell Activation.

Dr. Janeway's laboratory has extensively analyzed T cell activation by monoclonal anti–T cell receptor

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antibodies. This has shown that crosslinking of and conformational change in the T cell receptor both contribute to T cell activation. Antibodies can crosslink the T cell receptor without activating the cell, and Fab fragments of activating antibodies do not crosslink the T cell receptor and therefore do not activate the T cell. The two antibodies given together activate the T cell effectively. Furthermore, antibodies that bind effectively to the T cell receptor may or may not induce a physical association of the T cell receptor with CD4.

VI. A New Model for T Cell Repertoire Selection.

Based on these findings, Dr. Janeway has proposed that positive selection may involve either rare self peptides bound to self MHC molecules or MHC molecules alone on thymic epithelium. Binding of a T cell receptor to a rare self peptide could induce conformational change without crosslinking. MHC alone, together with co-ligands and co-receptors, could stabilize crosslinks without generating the high-affinity binding leading to conformational change that the true ligand for the T cell receptor induces. Either state could initiate the signal for positive selection.

These models can be tested by further analysis of self peptides on thymic epithelium, using new monoclonal antibodies being developed in Dr. Janeway's laboratory, and by examining the signals transduced in T cells by crosslinking or by conformational change alone. These studies will form the focus of research in Dr. Janeway's laboratory in the coming year. In addition, the molecular basis of self and non-self MHC recognition, of self peptide binding to class II MHC molecules, and of spontaneous diabetes mellitus are subjects of active research.

Dr. Janeway is also Professor of Immunobiology at the Yale University School of Medicine and of Biology at Yale University.

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T CELL SPECIFICITY

JOHN W. KAPPLER, PH.D., *Investigator*

Most T cells use clonally variable receptors made up of two chains, α and β, to recognize antigen. Functional genes for each of these chains are constructed by rearrangements of one each of a number of segments, by mechanisms that are similar to those used for immunoglobulin genes. In the mouse and human, chain genes can use 1 of ~50 Vαs and 1 of ~50 Jαs. In the mouse, β-chain genes can be constructed from 1 of ~20 Vβs, 1 of 2 Dβs, and 1 of 12 Jβs. The figures are similar in humans, with the exception that there are ~50 Vβ genes per haploid genome in this species. Additional variability between receptors on different T cells occurs because of the introduction or deletion of a number of bases into α and β genes at the points of V-J, D-J, or V-D joining.

The work of a number of laboratories has suggested that binding of a particular T cell receptor to its ligand [a peptide fragment of antigen bound to a cell surface major histocompatibility complex (MHC) protein] requires a contribution to binding of all the variable elements of the receptor, including Vα, Vβ, and Jα. Because any given combination of variable elements will be rare, T cells responding to most antigen-MHC complexes in a naive animal are rare, and primary responses to most infections are consequently small.

Some years ago, work from this laboratory demonstrated a surprising exception to this rule: in some cases Vβ alone could determine the specificity of T cells for certain antigens, almost regardless of the other variable elements of the receptors expressed on these cells. The antigens in question were called superantigens, because they stimulate almost all T cells bearing a particular Vβ(s), activate a relatively large percentage of all T cells, and give rise to enormous responses, even in a naive animal. For example, some mice express the superantigen Mls-2^a. This antigen stimulates nearly all T cells bearing Vβ3, providing these cells are derived from a mouse that does not itself express Mls-2^a. Consequently, T cells from Mls-2^a–negative animals, which are usually ~5% Vβ3^+, respond strongly to Mls-2^a–bearing cells from other animals. T cells from mice that themselves express Mls-2^a do not respond in this fashion, because the potentially self-reactive Vβ3-bearing cells are eliminated during development in the thymus.

So far about eight different superantigens have been found encoded in the genomes of various laboratory mice. The most biologically significant consequence of these superantigens to the mice that express them is the fact that they cause clonal deletion of the T cells with which they can interact. In some cases this amounts to deletion of a sizable percentage of the total T cell repertoire. Mls-1^a, for example, has been shown by this laboratory to delete Vβ8.1^+ T cells and by others to delete Vβ6- and Vβ9-bearing cells, ~22% of all T cells in the mouse.

Why do mice express these deleting superantigens? In an attempt to deal with this question, Dr. Kappler and his colleagues analyzed wild mice to find out if these animals, which presumably have to deal with the full gamut of mouse pathogens, express T cell–deleting superantigens. In collaboration with Drs. Edward Wakeland and Wayne Potts (University of Florida) 42 wild mice trapped near Gainesville were examined. Vβ-reactive, T cell–deleting superantigens were found in nearly all the animals. Some of the animals were also homozygous for a large deletion at their Vβ loci; only 10 of all mouse Vβ genes were present in these animals. Thus T cell deletion via Vβ is not a rare event in the wild. On the contrary, such deletion is almost universal; the strategy adopted in wild mice seems designed to cause a limited expression of Vβs in any given animal and to permit different animals to express different spectra of Vβs.

At an HHMI sponsored workshop in 1987 on the *Mls* locus, Dr. Steven Buxser presented data on the curious properties of a collection of toxins produced by *Staphylococcus aureus*. At the time these toxins were known to bind MHC class II proteins, and as such a complex to be powerful T cell stimulants in mice and humans. They were also known to cause several diseases in humans, including food poisoning, toxic shock, and scalded skin syndrome. This laboratory tested these toxins for their ability to stimulate T cells bearing different Vβs in mice and humans and found that in both species each toxin acted in a Vβ-specific way. Staphylococcal enterotoxin B, for example, stimulates mouse T cells bearing Vβ3, -7, -8.1, -8.2, and -8.3. The toxin responsible for toxic shock in humans stimulates mouse T cells bearing Vβ15, -10, and -3 and human T cells bearing Vβ2. Thus in every respect the staphylococcal toxins appear to be bacterial superantigens, analogous to the Mls-like superantigens made by mice themselves.

In collaboration with Dr. Brian Kotzin at the Na-

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tional Jewish Center, this laboratory has recently studied several patients who had been hospitalized with toxic shock. In a number of these patients, levels of Vβ2-bearing cells were significantly elevated, suggesting that the stimulatory properties of the toxins demonstrated in the test tube had consequences for the whole individual infected with an organism producing such a toxin.

Thus some or all of the pathological effects of these toxins may be due to the fact that they stimulate a sizable portion of all T cells in a given individual. The responding T cells will produce a collection of lymphokines, including interleukin-2, cachectin, and interferon-γ, which are known to have toxic effects when given in large amounts. Pathology may therefore be due to the T cell products and not directly to the toxins themselves. Wild mice may express particular superantigens and thus delete T cells bearing particular Vβs, in an attempt to preempt attack by particular toxins that may be produced by the flora in their environment, a suggestion that will be tested by further experiment.

Dr. Kappler is also Member of the Department of Medicine at the National Jewish Center for Immunology and Respiratory Medicine and Professor of Microbiology, Immunology, and Medicine at the University of Colorado Health Sciences Center.

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MOLECULAR GENETICS OF LYMPHOCYTE DEVELOPMENT

STANLEY J. KORSMEYER, M.D., *Associate Investigator*

The research effort in Dr. Korsmeyer's laboratory is focused on the molecular genetics of lymphoid differentiation and the corresponding lymphoid neoplasms. The immunoglobulin (Ig) and T cell receptor (TCR) genes undergo programmed rearrangements and deletions during normal development and are the sites of interchromosomal translocation introducing new proto-oncogenes in lymphoid tumors.

I. t(14;18) Lymphomas and the *Bcl-2* Gene.

A. Bcl-*2 regulation and deregulation*. The chromosomal breakpoint of the t(14;18) that typifies follicular B cell lymphoma juxtaposes a new putative proto-oncogene, *Bcl-2*, with the Ig heavy-chain locus. The t(14;18) generates chimeric *Bcl-2*–Ig RNAs that are excessive in these mature B cells. Monoclonal antibodies generated to the *Bcl-2* protein indicate that levels of this membrane-associated intracellular protein are also increased in lymphoma cells. A predominant mechanism for the deregulation of the *Bcl-2*–Ig fusion gene proved to be novel. Despite the substitution of an Ig 3′ end, the mRNA stability was not altered. However, the substitution of an Ig for the *Bcl-2* 3′ end has a dramatic effect on the processing or transport of this message. A series of chimeric gene constructs have confirmed that the *Bcl-2*–Ig fusion mRNA is much more efficiently handled in B cells than the *Bcl-2* message.

B. *Functional role of* Bcl-*2*. The finding that *Bcl-2* was juxtaposed with the Ig locus in follicular lymphoma but not in normal cells suggested it functioned as a proto-oncogene. Several approaches were undertaken to evaluate the oncogenic activity of *Bcl-2*. Retroviral expression vectors were used to introduce deregulated *Bcl-2* into B lymphoblastoid lines. Deregulated *Bcl-2* consistently produced a three- to fourfold increment in clonogenicity. Moreover, *Bcl-2* proved capable of complementing an exogenous *myc* gene present within lymphoblastoid lines, augmenting both clonogenicity and tumorigenicity. Retroviral *Bcl-2* was introduced into a variety of interleukin-dependent lines, to determine if *Bcl-2* was directly involved in a growth factor pathway. Overexpressed *Bcl-2* could not eliminate the long-term need for any interleukin. Instead, *Bcl-2* extended the short-term survival of interleukin-deprived cells. Deregulated *Bcl-2* did not influence cell cycle progression, while its predominant effect was to delay the onset of cell death. This effect was manifest in interleukin-3 (IL-3)-deprived prolymphocytes, promyelocytes, and mast cells but not IL-2– or IL-6–dependent cells. Thus *Bcl-2* interferes with cell death, but in a cell-type and/or factor-restricted fashion.

C. Bcl-*2–Ig transgenic mice*. The most comprehensive and unrestricted test of a gene's transforming capacity is to place it transgenically. Dr. Korsmeyer and his colleagues designed *Bcl-2*–Ig minigenes recreating the fusion gene found at the breakpoint. These minigenes were placed into the germline of mice to assess the prospective effects of the t(14;18) during development. The transgene demonstrates a lymphoid pattern of expression and uniformly results in an expanded follicular center cell population. Hyperplastic splenic follicles coalesce to form massive regions of white pulp resembling early splenic follicular lymphoma. Mice also develop regional lymphadenopathy with abnormal cellular infiltrates. The expanded lymphoid compartment consists of B220, IgM/IgD mature B cells. Provocatively, the *Bcl-2*–Ig transgene confers a long-term survival advantage to these B cells. *Bcl-2*–Ig transgenic mice document a prospective role for the t(14;18) in B cell growth and the pathogenesis of follicular lymphoma.

II. Translocations in T Cell Acute Lymphoblastic Leukemia Juxtapose New Putative Proto-oncogenes with the δ TCR Locus.

A. *t(11;14)(p15;q11) breakpoint and deregulated* Ttg-*1*. The most common translocations in T cell neoplasms involve 14q11. Many of these T cell acute lymphoblastic leukemias (ALLs) are immature in phenotype, lacking αβ TCRs and at times CD3. Dr. Korsmeyer and his colleagues reasoned that such ALLs would have translocations involving the δ TCR. This made it possible to obtain chromosomal breakpoints for the other partners of 11p13, 11p15, and 10q24 involved in ALL. For example, the t(11;14)(p15;q11) breakpoint results from an inadvertent TCR recombinase-mediated break at 11p15 that recombines with the δ TCR during its assemblage at a pre–T cell stage. The der (11) breakpoint resembled a coding joint with 11p15 in-

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troduced 5′ to a D1/D2/Jδ1 intermediate rearrangement. Symmetrically, the der (14) breakpoint corresponded to a perfect signal joint between the Dδ1 heptamer signal and a fortuitous heptamer signal from 11p15. Unlike other translocations, this event activates multiple, apparently distinct transcripts from both sides of the der (14) breakpoint. The analysis of one deregulated gene (*Ttg*-1) that encodes a 156–amino acid protein containing internal repeats with zinc finger motifs has been completed.

B. *t(10;14)(q24;q11) breakpoint and deregulated* Tcl-*3*. Similarly, this translocation in T-ALL introduces chromosome segment 10q24 into an intermediate δ TCR rearrangement at 14q11. A locus distinct from terminal deoxynucleotidyl transferase was found near the clustered breakpoint region on 10q24. Evolutionarily conserved regions surrounding the 10q24 breakpoint were examined for transcriptional activity. A locus on the der (14) chromosome is markedly deregulated, resulting in abundant amounts of a 2.9 kb mRNA from the candidate proto-oncogene *Tcl*-3.

III. Mapping and Walking Within Human Chromosome Segments Utilizing Yeast Artificial Chromosome Clones.

The vast majority of the chromosomal aberrations associated with cancer or developmental syndromes are located a considerable distance from known genes. This observation emphasizes the need to develop technologies that bridge the gap between cytogenetic and routine phage and cosmid cloning. Well-characterized large genomic clones obtained from yeast artificial chromosome (YAC) libraries provide the framework to localize genes and approach genetic disease. Universally applicable approaches to establish authenticity, localize and orient internal genes, map restriction sites, and rescue the distal ends of large human genomic DNA inserts were developed. Human chromosome segment 18q21.3 was selected as a model. Molecular cloning of this segment was initiated by characterizing three plasminogen activator inhibitor-2 (PAI-2) clones (290, 180, and 60 kb in size) isolated from a YAC library. Comparison of YAC and bacteriophage λ genomic DNA clones confirmed the fidelity of the PAI-2 locus. Detailed rare cutting restriction maps could be generated utilizing ramped contour-clamped homogeneous electric field electrophoresis. The PAI-2 locus could be located and oriented within the YACS. Moreover, both left and right ends of the YAC genomic DNA inserts were rescued by amplifying circularized cloning sites with an inverted form of the polymerase chain reaction. These unique terminal genomic DNA fragments were used to rescreen the YAC library and isolate overlapping clones that extend the map. These approaches will enable neighboring loci to be linked definitively and establish the feasibility of using YAC technology to clone and map chromosomal segments.

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REGULATION OF GENE EXPRESSION DURING CELL DIFFERENTIATION AND ACTIVATION

JEFFREY M. LEIDEN, M.D., PH.D., *Assistant Investigator*

Dr. Leiden's objective is to understand better the molecular mechanisms that regulate gene expression during the processes of cellular activation and differentiation. Toward this end, Dr. Leiden and his colleagues have been studying transcriptional regulation in two different experimental systems, the human T lymphocyte and the mouse myocyte. The studies of human T cells have focused on the regulation of the T cell receptor (TCR) α and β genes during T cell differentiation and the regulation of the 4F2 heavy-chain (4F2HC) gene during T cell activation. The studies of mouse myocyte differentiation have utilized the skeletal muscle troponin C (sTnC) and cardiac troponin C (cTnC) genes as models of developmentally regulated, lineage-specific myocyte gene expression.

I. Regulation of Human TCR α Gene Expression During T Cell Ontogeny.

A. *Identification and characterization of a T cell–specific transcriptional enhancer 3′ of the Cα gene in the human TCR α locus.* Human T lymphocytes can be divided into two distinct subsets, based on their cell surface expression of antigen receptor molecules. The majority of peripheral blood T cells, including most cells of the helper and cytotoxic phenotypes, express a CD3-associated α/β TCR. A second smaller subset of T cells of unknown function express a CD3-associated γ/δ TCR. Although a great deal has been learned about the structure of the four TCR genes, little is understood about the molecular mechanisms that regulate the rearrangement and expression of these genes during T cell ontogeny.

Dr. Leiden's laboratory has identified a transcriptional enhancer element located 4.5 kilobases (kb) 3′ of Cα in the human TCR α-chain locus. This enhancer is necessary for transcription from a TCR Vα promoter and is also active upon the minimal SV40 promoter in TCR α/β^+ T cells. However, it is inactive in human TCR γ/δ^+ T cells, B cells, and fibroblasts. The enhancer has been localized to a 116 bp *Bst*XI/*Dra*I restriction enzyme fragment that lacks immunoglobulin octamer and κB enhancer motifs but does contain a consensus cAMP-responsive element (CRE). DNase I footprint and electrophoretic mobility shift analyses demonstrated that the minimal enhancer contains two binding sites for Jurkat nuclear proteins. One of these sites corresponds to the CRE, while the other does not correspond to a known transcriptional enhancer motif. These data support a model in which TCR α gene transcription is regulated by a unique set of cis-acting sequences and trans-acting factors that are differentially active in cells of the TCR α/β^+ lineage. Activation of the TCR α enhancer may be a key step in determining the differentiation of TCR α/β^+ T cells. In addition, the TCR α enhancer may play a role in activating oncogene expression in T lymphoblastoid tumors, which have previously been shown to display chromosomal translocations into the human TCR α locus. Ongoing studies are aimed at determining the importance of each of the nuclear protein-binding sites in enhancer function and elucidating the molecular mechanisms responsible for the preferential activity of the enhancer in TCR α/β^+, as compared with TCR γ/δ^+, T cells. Dr. Leiden's laboratory is also in the process of cloning the DNA-binding proteins that regulate the activity of this enhancer.

B. *Molecular cloning of CREB-2, a novel CRE-binding protein that interacts with the human TCR α transcriptional enhancer.* The TCR α enhancer contains a nuclear protein-binding site, Tα1, which includes the palindromic sequence TGACGTCA that is identical to the sequence motif that confers cAMP responsiveness on multiple eukaryotic promoters (CRE). Using electrophoretic mobility shift analyses, Dr. Leiden's laboratory has shown that a CRE-binding protein (CREB) present in T cell nuclear extracts binds specifically to the Tα1 site of the TCR α enhancer. By using a radiolabeled TCR α CRE probe to screen a Jurkat λgt11 cDNA expression library, they have identified a human cDNA clone that encodes a novel protein that binds specifically to the TCR α enhancer CRE (CREB-2). The 351–amino acid CREB-2 protein contains a carboxyl-terminal leucine zipper motif and an adjacent basic domain that are structurally related to similar domains in the c-*jun* and c-*fos* 12-*O*-tetradecanoylphorbol-13-acetate (TPA) response element (TRE)-binding proteins, as well as to the previously described CRE-binding proteins, CREB and CRE-BP1. Comparison of the basic domains of the known TRE- and CRE-binding proteins has allowed the identification of three amino acid residues that may account for the different binding specificities of these two related families of transcription factors.

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These results suggest that a family of distinct proteins sharing a common structural motif may regulate the diverse transcriptional effects mediated by CREs. Dr. Leiden's laboratory is studying the interaction of CREB-2 with other recently described CRE-binding proteins and testing the role of specific amino acids in the basic domains of CRE- and TRE-binding proteins in determining the specificity of DNA binding of these two families of transcriptional regulators.

II. Structure, Function, and Expression of the Cardiac Troponin C Gene.

Cardiac troponin C (cTnC) is the calcium-binding subunit of the myofibrillar thin filament that regulates excitation-contraction coupling in cardiac muscle. Dr. Leiden's laboratory has utilized a novel polymerase chain reaction–cloning procedure to isolate cDNA clones encoding murine cTnC. Murine cTnC is a 161–amino acid polypeptide that has been highly conserved during evolution. Dr. Leiden's laboratory has shown that the cTnC gene is a member of a multigene family and is expressed in murine cardiac and slow skeletal muscle but not in fast skeletal muscle, nor in neonatal or adult brain, kidney, lung, liver, or testis. In addition, although the cTnC gene is not expressed in murine C2C12 myoblasts, differentiation of these cells into myotubes was shown to result in a dramatic induction of cTnC gene expression. A full-length cTnC genomic clone was isolated from a murine genomic library. The cTnC gene is 3.4 kb long and is composed of six exons. Analysis of the 5′-flanking region of the gene revealed the presence of a consensus TATA box 24 bp 5′ of the transcription start site. However, despite the finding that the gene is expressed only in cardiac and slow skeletal muscle, it lacks the previously described CArG and M-CAT sequence motifs, which are involved in regulating the expression of a number of other myofibrillar genes. These results demonstrate that cTnC gene expression is developmentally regulated and lineage specific. Dr. Leiden and his colleagues have also defined an *in vitro* system, the C2C12 myoblast, which can be used in transfection studies designed to identify the cis-acting regulatory elements and trans-acting factors that control cTnC gene expression during myocyte differentiation. It is hoped that such studies will lead to the identification of novel cardiac-specific transcriptional regulatory factors.

Dr. Leiden's laboratory has also recently designed a prokaryotic expression system that has allowed the production and purification of large amounts of recombinant cTnC protein. This purified recombinant protein has been used to produce monoclonal antibodies specific for cTnC. Because cTnC is a relatively small and abundant protein that is expressed in a cardiac-specific fashion, an ELISA assay based on these antibody reagents may be useful in the early diagnosis of patients suffering from acute myocardial infarction. A clinical trial of these antibodies is in progress.

Dr. Leiden is also Assistant Professor of Internal Medicine and of Microbiology and Immunology at the University of Michigan Medical School.

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PATHOGENETIC PROCESSES IN HUMAN IMMUNODEFICIENCY VIRUS INFECTION

DOROTHY E. LEWIS, PH.D., *Assistant Investigator*

The overall objective of the research being performed in Dr. Lewis's laboratory is to understand the biology of human immunodeficiency virus (HIV) infection. During the past year, several areas of investigation have been undertaken.

I. *In Vitro* Transmission of HIV: Derivation of $CD8^+$ HIV-infected Cell Lines.

Dr. Lewis originally demonstrated that $CD8^+$ cells from most asymptomatic HIV-infected individuals are both phenotypically and functionally abnormal. In the past year, an *in vitro* system to study the effect of HIV on $CD8^+$ cells has been explored. In this experimental system, HIV is transmitted via infected, irradiated cells to activated lymphocytes from the same individual. In 5–7 days, most of the $CD4^+$ cells are killed, concomitant with a burst in viral output. The cells remaining are predominantly $CD8^+$ cells, which have been maintained in interleukin-2 (IL-2)-containing medium for 4–5 months after initiation of cultures. The cells have been shown to be infected productively with HIV, as detected by p24 antigen production, *in situ* hybridization, and electron microscopy. The cells are T cells, as detected by anti-CD3 and T cell receptor (TCR) staining; most are DR^+ and $CD38^+$, but only a few are IL-2 receptor positive. CD57 is not detectable in these cell lines. Functionally the cells are not as responsive to T cell signaling, and, in most cultures, T cell activation has a detrimental effect on cellular viability. Experiments are in progress to determine whether a CD4 message exists in these cell lines and whether transmission of HIV requires CD4 or is mediated by some other molecule, such as lymphocyte function antigen 1 (LFA-1).

II. Anti-HIV Effect of Plant Phospholipids.

Dr. Lewis's laboratory also has examined the effects of certain soybean-derived phospholipids on HIV production *in vitro*. The compounds have proven effective in reducing HIV viral levels in tumor cell lines and in *in vitro*–infected human lymphocytes, as detected by p24 antigen ELISA. Unsaturation at the Sn 2 position of the fatty acid chain is required for activity. At high concentrations (100 μM), the compounds are toxic to cells *in vitro*; but at lower concentrations (25 μM), the compounds reduce HIV production 50–80%, with minimal toxicity. Cellular proliferation is slowed at these concentrations, but there is a preferential effect on HIV-infected cell lines. The compounds do not prevent syncytia formation and require several days to reach maximal effect. The compounds can be washed out, and viral production is still reduced up to four days after elimination of the compounds, suggesting a long-lasting effect. The compounds reduce HIV message production, as detected by *in situ* hybridization. The compounds do not inhibit the p24 antigen ELISA determination *per se*.

In preliminary *in vivo* animal toxicity tests, the compounds are not toxic in gram dosages. The compounds also have been shown to kill multiresistant tumor cells preferentially *in vitro*, presumably via accumulation of toxic fatty acid products after phosphorylase A_2 action. Experiments are in progress to determine the mode of action of these drugs on HIV production.

III. *In Situ* Hybridization Detection Using Confocal Microscopy.

Most investigators have suggested that very few transcriptionally active HIV-infected cells are present *in vivo*. This is based on *in situ* hybridization, using ^{35}S-labeled HIV RNA and conventional microscopy as the detection system. This is difficult to comprehend, because of the massive $CD4^+$ cellular depletion that occurs and the immunodeficiency that is probably responsible for most of the pathology. There are models to explain the depletion, including syncytia formation or cytotoxic T cell or ADCC (antibody-dependent cellular cytotoxicity) killing of infected cells; however, none of these mechanisms have been shown to be significant *in vivo*. Dr. A. S. Fauci has recently presented data from four acquired immune deficiency syndrome (AIDS) patients suggesting that 1 per 100 or 1 per 1,000 $CD4^+$ cells contain HIV DNA, as detected by polymerase chain reaction amplification. Dr. Lewis and her colleagues have coupled conventional ^{35}S-based RNA *in situ* hybridization with an alternative detection system, the confocal laser digital imaging microscope, which enhances *in situ* detection ~100-fold. The ability to focus the laser light enhances contrast so that the background, which is in the same plane as the cells, disappears and the reflectance image, which is above the plane of the

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cells and is created by the exposed silver grains, is absolutely clear-cut. In three experiments (using the National Institutes of Health resource facility in Madison, Wisconsin), Dr. Lewis and her colleagues have studied 55 HIV-infected individuals in blinded fashion with no knowledge of their clinical condition. Reproducibility of the measurement was confirmed in eight blinded, split-blood specimens. Of the 25 AIDS patients studied, ~50% had >1 transcriptionally active cell per 300 cells. More importantly, there was a strong negative correlation between *in situ* numbers and Karnofsky score (a measure of clinical well-being), which indicates that the presence of increased numbers of transcriptionally active HIV-infected cells is associated with declining clinical condition. This may indicate that viremia *per se* can account for the pathogenesis of HIV infection. Other parameters examined included CD4 numbers, time on azidothymidine (AZT) treatment, other hematologic variables, and CD8 cellular subsets. No correlation was observed between *in situ* number and CD4 cellular count. Increased percentages of $CD8^+$ $CD57^+$ cells correlated with increased numbers of transcriptionally active cells. Future experiments will examine larger numbers of individuals, measure p24 antigen levels as an indicator of viremia, and determine whether time on AZT treatment is significantly associated with levels of transcriptionally active cells.

Dr. Lewis is Assistant Professor of Microbiology and Immunology at Baylor College of Medicine.

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FUNCTION OF T CELL SURFACE GLYCOPROTEINS IN DEVELOPMENT AND RETROVIRAL ENTRY

DAN R. LITTMAN, M.D., PH.D., *Assistant Investigator*

The major interests of Dr. Littman's laboratory are 1) the mechanisms through which T cell surface molecules specify developmental pathways of antigen-specific T cells and 2) the cell surface receptors required for entry of human retroviruses into target cells. The studies with T lymphocytes are aimed at deciphering the functions of the CD4 and CD8 glycoproteins, cell surface molecules involved in T cell differentiation and T cell activation. Studies with retroviruses have focused on the molecular basis of the interaction between the envelope glycoprotein of the human immunodeficiency virus (HIV) and the viral receptor CD4. Ongoing studies are aimed at identifying a second cell surface molecule required for HIV entry and the receptor for the human T cell leukemia viruses (HTLV-I and HTLV-II).

I. Function of CD4 and CD8 in Thymocyte Differentiation.

The process of thymocyte differentiation yields mature T cells that are tolerant to self antigens yet react to foreign antigens complexed to host major histocompatibility complex (MHC) molecules. During this process, clonally distributed T cell receptors (TCRs) interact with MHC molecules on specialized cells within the thymus. These interactions result in elimination of clones with TCRs that react to self antigens complexed to host MHC molecules (thymic tolerance) and expansion of clones with TCRs specific for foreign antigens complexed to host MHC molecules (positive selection). In the course of clonal expansion, thymocytes that express both the CD4 and CD8 cell surface glycoproteins (double positive cells) give rise to mature cells that express either one or the other molecule: the $CD4^+$ T cells, primarily helper cells whose T cell antigen receptors are specific for class II MHC molecules, and $CD8^+$ cells, almost exclusively cytotoxic cells with receptors recognizing class I MHC molecules. There is mounting evidence that signals transmitted through the CD4 and CD8 molecules are important in clonal deletion and expansion and in determining the developmental pathway of double positive thymocytes. Research in Dr. Littman's laboratory has focused on the ligand-binding properties of the CD4 and CD8 molecules and on the role of an intracellular lymphocyte-specific tyrosine kinase, $p56^{lck}$, in transducing signals received by CD4 and CD8.

The CD4 and CD8 molecules have been shown to bind directly to MHC-II and MHC-I molecules, respectively. In collaboration with Dr. Peter Parham (Stanford University), Dr. Littman's laboratory has shown that CD8 binds to a membrane-proximal domain of the MHC-I molecule. Because this domain is distinct from that recognized by the TCR, a single MHC molecule can crosslink CD8 and the TCR, thus initiating a specific signal that may be important in development of the thymocyte. Point mutations within the membrane-proximal domain of MHC-I result in loss of binding of CD8 but do not affect binding of peptides or recognition by the TCR. MHC-I molecules bearing such mutations have been introduced into transgenic mice, allowing analysis of the role of CD8-MHC binding in tolerance induction and in selection of the TCR repertoire.

It has recently been demonstrated that both CD4 and CD8 are noncovalently associated with a cytoplasmic protein tyrosine kinase, $p56^{lck}$, a member of the *src* family of kinases. This interaction may be important for transmembrane signal transduction in T cell activation, as well as in thymocyte differentiation. Recent work in Dr. Littman's laboratory has shown that this association is dependent on cysteine residues within a sequence shared by the cytoplasmic domains of both CD4 and CD8 and on a pair of cysteines in the amino-terminal domain of the kinase. Binding is disrupted by divalent metal cation chelators, suggesting that a metal coordination complex is critical in the interaction. Functional studies with antigen-specific T cell hybridomas indicate that the interaction of CD4 with $p56^{lck}$ is required for effective activation of the T cell. To study the importance of the interaction of CD4 or CD8 with $p56^{lck}$ during development, Dr. Littman and his colleagues, in collaboration with Dr. Roger M. Perlmutter (HHMI, University of Washington), have prepared transgenic mice expressing mutant CD4 molecules that do not bind the kinase. Development of thymocytes in these animals is being investigated. Since the absence of an intact endogenous CD4 or CD8 gene would greatly facilitate *in vivo* studies of mutant molecules, Dr. Littman's group is preparing mice that are defective in the expression of CD4 and CD8. The CD4 gene has been disrupted by homologous recombination in embryonal stem cells; these cells can now be injected into mouse blastocysts to yield chimeric ani-

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mals that transmit the mutant gene to their offspring.

II. Interaction of Retroviruses with Receptors.

Entry of HIV into target cells involves high-affinity binding of the viral envelope glycoprotein to CD4, fusion of the viral membrane to cellular membranes, and uncoating of the virus. Work in Dr. Littman's laboratory has focused on the role of CD4 in viral binding and, more recently, on defining molecules other than CD4 that are required for entry. These studies have been extended to develop retroviruses that can be targeted to specific tissues and that can be used to isolate genes encoding the cell surface virus receptors.

HIV displays enormous polymorphism, making the prospect of developing immunological agents to combat infection quite daunting. The region of the virus that is exempt from significant variability is the domain of the envelope glycoprotein, gp120, that interacts with the receptor, the CD4 glycoprotein. This interaction is likely to be the key to a number of potential antiviral therapies. Amino acid residues in CD4 that interact with gp120 have been identified by analyzing the ability of numerous CD4 point mutants to bind to gp120 and to anti-CD4 monoclonal antibodies. Two regions predicted to lie in close proximity to each other in the immunoglobulin-like amino-terminal domain of CD4 are involved in HIV binding. Monoclonal antibodies were found to bind in the same region, but none of the antibodies coincided with gp120 in their requirements for binding to CD4 mutants. This approach may facilitate screening of anti-CD4 monoclonal antibodies that have binding sites for CD4 similar to the binding site on gp120; such antibodies may be useful for preparing anti-HIV vaccines. The design of drugs that block the virus-receptor interaction will be facilitated by an understanding of the three-dimensional nature of the binding. To this end, soluble complexes of CD4 and gp120 have been prepared for crystal analysis in collaboration with Dr. Robert Stroud (University of California at San Francisco).

A number of observations suggest that a molecule other than CD4 is required for fusion of HIV to cellular plasma membranes. The same or other molecules may also be sufficient for viral entry in some cell lines that lack CD4. To identify other genes required for HIV entry, Dr. Littman and his colleagues have prepared retroviruses containing the HIV envelope glycoprotein and encoding selectable markers; cells that have intact receptors for HIV can be selected by virtue of their ability to internalize these viruses. This approach is also being used to prepare viruses bearing the envelope glycoprotein of HTLV-I; these viruses are being employed to identify the receptor for HTLV-I and HTLV-II.

Dr. Littman is also Assistant Professor of Microbiology and Immunology and of Biochemistry and Biophysics at the University of California at San Francisco.

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DEVELOPMENTAL BIOLOGY OF T LYMPHOCYTES

DENNIS Y. LOH, M.D., *Associate Investigator*

Thymus-derived lymphocytes (T cells) possess three characteristic features. 1) Each T cell has a unique pair of cell surface receptors, the T cell receptors (TCRs), by which antigens are recognized. 2) Unlike the immunoglobulin molecule, the antigens can be recognized only in the context of the self MHC (major histocompatibility complex) products, a phenomenon known as MHC restriction. 3) Self-reactive T cells must be eliminated or at least rendered nonfunctional if the organism is to avoid autoimmunity and self organ rejection. The developmental processes by which a mature T cell arises are the focus of Dr. Loh's research. This includes the analysis of TCR gene rearrangements and expression and subsequent cell fate determination as a result of TCR-MHC interaction in the thymus.

I. Positive-Selection Model of MHC Restriction.

MHC restriction can be described as skewing of the peripheral T cell repertoire toward recognition of antigens in the context of self MHC molecules; this process has been shown to occur in the thymus during development. To analyze how skewing occurs, Dr. Loh introduced a pair of TCR genes from an allogeneic cytotoxic T cell clone (2C) into mouse embryos to create TCR transgenic mice. Clone 2C was originally derived from an H-2^b animal, and its specificity is directed against an element in H-2^d. In H-2^b transgenic mice, the majority of peripheral T cells expressed the 2C TCR, and its expression was predominantly restricted to the $CD4^-CD8^+$ population. Surprisingly, when these mice were bred to H-2^s or H-2^k backgrounds, the transgenic TCR-bearing cells failed to emerge in the periphery. In F1 animals such as H-2^{bxs} or H-2^{bxk}, T cells bearing transgenic TCR were allowed to emerge into the periphery, eliminating clonal deletion as a mechanism for the lack of transgenic T cells in homozygous H-2^s or H-2^k mice. Thus an element present in the H-2^b mice may be responsible for positively selecting the 2C TCR in conjunction with the CD8 molecule in the thymus.

Subsequently, using recombinant inbred mice, the positively selecting element in H-2^b has been mapped to the H-2K^b locus. To prove that the molecular basis of positive selection depends on the K^b molecule itself, the TCR transgene-bearing mice were backcrossed to bm1, a congenic mouse strain bearing a mutant K^b molecule. It has been shown that the three amino acid differences between K^b and K^{bm1} molecules is sufficient to abrogate positive selection. These data strongly suggest that a T cell bearing a surface TCR must interact productively with the MHC molecules displayed in the thymus before they are allowed to emerge as functionally and phenotypically mature T cells. This system will allow Dr. Loh to study T cell development as a function of molecular interactions between the TCR/CD8 and MHC in the thymus. Incidentally, these data are directly relevant to transplantation rejection phenomenon, since they explain the origin of alloreactive T cells in terms of cross-reactivity resulting from positively selected self MHC-restricted T cells.

II. Mechanism of Self-Tolerance to Antigens Known to be Present in the Thymus.

To test the mechanism of self-tolerance development, the 2C transgenic mice were backcrossed to H-2^{bxd} and H-2^d backgrounds. In the periphery of these mice, functional T cells bearing the 2C TCR were deleted. Analysis of the thymocytes revealed that this elimination of the autoreactive T cells was taking place at or before the $CD4^+CD8^+$ stage in thymocyte development. These findings are consistent with the clonal deletion model of tolerance development against antigens known to be present in the thymus. In addition, immunocytochemical analysis of the thymus undergoing clonal deletion revealed that the deletion was taking place in the thymic cortex. These data also suggest that a distinct positive-selection step is not required for negative selection to mediate clonal deletion, as exemplified in the H-2^d mouse.

III. Mechanism of Self-Tolerance to Antigens Whose Expression is Restricted to be Extrathymic.

To examine the effects of aberrant expression of class II MHC proteins expressed exclusively in the periphery on tolerance development, Dr. Loh and his colleagues produced transgenic mice expressing the I-A^d genes under control of the pancreatic elastase promoter. Such transgenic mice express I-A^d exclusively on exocrine pancreas, without expression in thymus or by lymphocytes. No spontaneous development of autoimmune reactivity toward exocrine pancreas was found in transgene-

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expressing mice of an H-2^b background, even though such mice could produce *in vitro* allogeneic responses against I-A^d. When T cells from nontransgenic and transgenic H-2^b mice were activated *in vitro* by I-A^d allogeneic stimulator cells and transferred to transgenic mice, an intense, destructive lymphocytic infiltrate specific for exocrine pancreas developed. These findings suggest that aberrant class II MHC expression alone does not trigger autoimmune reactions, nor does it cause clonal deletion of functional autoreactive cells. Rather, the unresponsiveness to allogeneic class II MHC may result from the inability of exocrine pancreas to initiate primary responses by T cells.

The data obtained from the TCR and MHC transgenic mice strongly suggest that both clonal deletion and peripheral "anergy" may be operational *in vivo* to explain self-tolerance.

IV. Molecular Genetics of TCR β-Chain Genes.

To elucidate the basis for the tissue and developmental stage-specific expression of the TCR β-chain genes, Dr. Loh and his colleagues have continued to analyze the transcriptional apparatus. Both *in vivo* and *in vitro* analyses have been used to identify a regulatory region of the murine Vβ promoter. The results of transient transfection assays indicate that the dominant transcription-activating element within the Vβ8.3 promoter is the palindromic motif previously identified as the conserved Vβ decamer (AGTGACATCA). Elimination of this element, by linear deletion or specific mutation, reduces 10-fold the transcriptional activity from this promoter. DNase I footprinting, gel-mobility shift, and methylation interference assays confirm that the palindrome acts as the binding site of a specific nuclear factor. In particular, the Vβ promoter motif functions *in vitro* as a high-affinity site for a previously characterized transcription activator, ATF/CREB (activating transcription factor/cAMP-responsive element–binding protein). A consensus CRE, but not a consensus AP-1 site, can substitute for the decamer *in vivo*. These data suggest that ATF/CREB or related proteins activate Vβ transcription. Since the decamer motif has been found in other T cell relevant genes, such as terminal deoxynucleotidyl transferase and CD8, further work may elucidate the mechanism of coordinate gene expression in T cells.

Dr. Loh is also Associate Professor of Medicine, Microbiology and Immunology, and Genetics at Washington University School of Medicine and Assistant Physician at Barnes Hospital, St. Louis.

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T CELL DEVELOPMENT

PHILIPPA MARRACK, PH.D., *Investigator*

T cell development occurs most efficiently in the thymus. In this organ, precursor cells progress through a number of transformations, which are crucial to the eventual appearance of mature T cells that will be useful to the animal. These transformations include rearrangement and expression of the genes that code for the αβ or γδ receptors, which the cell will eventually bear, and induction of expression of a number of other genes that are required for the function of mature T cells, in particular CD4 and CD8. These events appear to be common to almost all precursors. Subsequently the developing αβ-bearing cell must go through two other tests, based on the specificity of its receptor: 1) positive selection for self-restriction [i.e., cells are picked out to mature if their αβ receptors will be able to recognize foreign antigens bound to self major histocompatibility complex (MHC) molecules] and 2) clonal deletion of potentially autoreactive cells. Only after a thymocyte has been successfully selected for self-restriction and has proved not to be self-reactive does it mature and become a functional peripheral T cell.

A few years ago this laboratory was the first to show that tolerance to self is achieved, in part, by clonal elimination of potentially self-reactive cells in the thymus. This was done in studies on the fate of T cells bearing particular Vαs as part of their αβ-receptors. In some cases the presence of a particular Vβ on the surface of a T cell is predictive of its reaction with a particular superantigen. T cells bearing Vβ3, for example, react with the mouse-expressed superantigens Mls-2^a or Mls-3^a. Expression in the animal of a particular superantigen caused the disappearance in the thymus of cells bearing the Vβ that this superantigen could stimulate. At the time, however, reagents were not available to allow a determination of exactly when in thymocyte development such deletion occurred.

Recently, in collaboration with Dr. Ralph Kubo (National Jewish Center for Immunology and Respiratory Medicine) a monoclonal antibody was developed against all mouse αβ-receptors that could be used in such an experiment. With this reagent it was shown that self antigens caused the disappearance of all mature, and about half the immature, potentially reactive thymocytes. This suggested that clonal deletion could occur when the target thymocyte was an immature, $CD4^+$, $CD8^+$, cortical thymocyte.

This discovery posed a new problem, because selection for self-restriction also occurs at this stage of thymocyte development. Because both positive selection and clonal deletion depend on reaction with the αβ-receptor, then both must be signaling the thymocyte through its receptor; thus the problem arises of how the thymocyte distinguishes between a selecting or a deleting signal. It has been suggested that the distinction depends on the cell with which the thymocyte is interacting, because different MHC-presenting cells might provide different secondary signals to the thymocyte. Interactions between receptor and MHC molecules on thymus cortical epithelial cells, for example, might lead to positive selection, whereas interactions between receptors and MHC on bone marrow–derived macrophages or dendritic cells might lead to clonal deletion. There is a good deal of evidence to support this notion. Other mechanisms have not, however, been excluded.

This problem has recently been tackled by members of this laboratory, in collaboration with Dr. Kubo and Dr. John Cambier (National Jewish Center for Immunology and Respiratory Medicine). Thymocyte development was studied *in vitro* in cultured intact thymus lobes from fetal mice. In such cultures thymocyte development proceeds normally, with the exception that the lobes gradually accumulate mature thymocytes and become depleted in precursor cells, because prothymocytes in the organ are not self-regenerating but are replenished in the intact animal by fresh precursors from bone marrow. Therefore, in such cultures, αβ-$receptor^-$, $CD4^-$, and $CD8^-$ precursors gradually disappear, giving rise to the αβ-$receptor^+$, $CD4^+$, $CD8^+$ intermediate cells and eventually to $CD4^+$ or $CD8^+$, single, positive, mature thymocytes. Addition of anti-αβ-antibody to such cultures caused the disappearance of all mature thymocytes and half of the immature $\alpha\beta^+$ cells; addition of an antibody to CD3, the complex of proteins with which αβ is associated on cell surfaces, led to the elimination of all $\alpha\beta^+$ cells, both mature and immature. Study of the ability of the different antibodies to induce increases in intracellular Ca^{2+} showed that anti-CD3 caused Ca^{2+} increases in all receptor-bearing cells, whereas anti-αβ caused increases in all mature thymocytes but only half of the immature $\alpha\beta^+$ cells, the half that were eliminated by anti-αβ-antibody. It is likely

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that the observations with anti-αβ-antibody are analogous to the reactions between thymocytes and natural antigens, which must also engage the cell via αβ, not CD3.

Several conclusions were drawn from these experiments. First, the studies showed that the immature pool of $\alpha\beta^+$ thymocytes, formerly thought to be a single pool of cells, consisted of two different populations, only one of which could be deleted by receptor ligation. It is therefore tempting to predict that the forces of positive selection might act on the nondeletable pool. Second, these data support the hypothesis that increases in intracellular Ca^{2+} are part or all of the signal for thymocyte death. Third, different stages of thymocyte development seem to be defined by different degrees of coupling between αβ-receptor proteins and the proteins with which they are associated, those of CD3, which are thought to transduce αβ-initiated signals to the cell bearing them.

Dr. Marrack is also Member of the Department of Medicine at the National Jewish Center for Immunology and Respiratory Medicine and Professor in the Departments of Biochemistry, Biophysics, and Genetics, and of Microbiology, Immunology, and Medicine at the University of Colorado Health Sciences Center.

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MECHANISMS OF ALLERGIC HYPERSENSITIVITY

CHARLES W. PARKER, M.D., *Investigator*

The importance of genetic influences on human respiratory allergy has been known for more than 60 years and indeed was suggested long before that. Human beings show marked differences in their immunoglobulin E (IgE) levels, which are an important factor in determining whether they develop respiratory allergy. IgE-mediated allergy is the most common genetic problem in the world, but little is known about its genetic control.

Dr. Parker and his colleagues have been studying the genetic control of IgE production in humans and inbred mice. Up to 10-fold variations in basal levels of IgE in serum have been found in different mouse strains. Two independently segregating genes that selectively regulate IgE levels have been identified. One of the genes is X-linked, and the other is autosomal. The autosomal gene has at least three alleles. In F1 hybrids and their backcrosses to parental strains, alleles for the intermediate and low IgE phenotypes are dominant over the high serum IgE phenotype. The gene has been mapped to chromosome 8. This localization excludes major histocompatibility antigens and structural genes for immunoglobulins and hormonal regulators of IgE production, such as interleukin-4 and interferon-γ, as the site of genetic control in the mouse. *In vitro* studies of IgE synthesis and finer genetic mapping studies are under way to localize and identify the gene and its protein product more precisely, determine their structures, and elucidate their role in the regulation of IgE production. Breeding studies are being undertaken to determine if the X-linked and autosomal genes can complement one another to produce still higher basal IgE levels. IgE levels will also be examined in animals stimulated with anti-IgD antibodies or infection with a helminthic parasite to determine if the genes controlling basal and stimulated IgE levels are the same.

Another major emphasis in Dr. Parker's laboratory has been on hormonal effects in autoimmunity. Of the common autoimmune conditions affecting human beings, ankylosing spondylitis is much more common in males than females, whereas systemic lupus erythematosus (SLE), rheumatoid arthritis, Sjögren's syndrome, Hashimoto's disease, Graves' disease, and pernicious anemia are substantially more frequent in females. Although the reason for the male or female predilection in most of these diseases is uncertain, in SLE, estrogenic hormones appear to be involved directly. Kunkel and his colleagues showed about a decade ago that estrogen metabolism *in vivo* is altered in lupus patients and their relatives, with increased hydroxylation of estradiol at position 16 (compared with controls with and without other diseases). Previous studies with SLE have indicated that suppression of estrogenic effects by castration or administration of male hormones may considerably ameliorate the disease. However, the basis for the estrogen effect is uncertain, and less-radical means of treatment are needed.

Dr. Parker and his colleagues have been studying estrogen metabolism in the NZB/NZW and NZB/SWR mouse hybrid models for SLE. These animals spontaneously develop autoantibodies and nephritis and die prematurely of renal failure. Each of the parental strains provides several genes that predispose to the disease. Hybrids of NZB mice with other strains are much less affected by autoimmune manifestations. Both of the susceptible hybrids show earlier and more marked manifestations of disease in females than males, suggesting their usefulness as an animal model to elucidate the role of sex hormones in this disease. The increased susceptibility of females of these hybrids to SLE appears to involve genes from the SWR and NZW strains rather than the NZB strain. Dr. Parker and his colleagues have studied the estrogen 16-hydroxylase P450 enzyme in the liver of various mouse strains. This enzyme produces multiple products and is highly polymorphic. A shared polymorphism of the enzyme in the NZW and SWR strains, which is lacking in seven of the eight other strains studied, including the NZB strain, has been identified. The next step is to determine if the alleles for the NZW and SWR enzyme are correlated with the presence of disease in NZB/W X NZB backcrosses, which have inherited other genes predisposing to the disease. If a relationship can be established, the structure and regulation of this enzyme will be studied, in an attempt to determine how and at what level the alteration in estrogen metabolism affects immune responsiveness.

A new protein-sequencing method is also being developed. An increase in sensitivity of at least 10- to 100-fold over previous methods is anticipated. Because there are many proteins that remain to be

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defined structurally and many of them are produced in limited quantities, the method should find widespread application.

Dr. Parker is Professor of Medicine and of Microbiology and Immunology at Washington University School of Medicine.

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MOLECULAR NEUROIMMUNOLOGY

DONALD G. PAYAN, M.D., *Assistant Investigator*

Dr. Payan is examining the biochemical activities of neuropeptides and neural mediators active in various phases of inflammation. The principal ongoing experimental effort is to analyze the proliferation and growth-promoting properties of these factors, through a detailed study of their receptors and associated signaling pathways. To this end, the receptor for the neuropeptide substance P (SP) and the signaling pathways associated with the histamine receptor are being actively investigated. The principal achievements of Dr. Payan and his colleagues in the past year include 1) a detailed analysis, using antipeptide antibodies, of the epitopes on the SP receptor that may be coupled to peptide-induced signaling; 2) the solubilization and characterization of the histamine (H_1) receptor from cultured smooth muscle cells; and 3) the study of the multiple signaling pathways of H_2 receptors.

I. Tachykinin Receptor Cross Talk.

Dr. Payan and his colleagues chemically synthesized peptides that correspond to the four extracellular domains of the predicted bovine substance K (SK) receptor protein and raised specific polyclonal antibodies against these peptides. These antibodies were then tested for both functional and structural recognition of epitopes on the SP receptor on rat AR42J pancreatic cells, which express only SP, and not SK, receptors. Antibodies directed against the second and fourth external domains of the predicted SK receptor specifically recognized a 58 kDa protein on AR42J cells, of similar molecular weight to that of Bolton-Hunter ^{125}I-labeled SP ([^{125}I]BH-SP)-crosslinked membrane proteins of AR42J cells and the previously reported SP receptor on human IM9 lymphoblasts. These antibodies also inhibited specific [^{125}I]BH-SP binding on both AR42J and IM9 cells. Furthermore, these antibodies also inhibited SP-induced mobilization of intracellular Ca^{2+} ($[Ca^{2+}]_i$) on AR42J cells. These data suggest homologies between the extracellular structures of the SP and SK receptors and imply that the second and fourth domains of these receptors may share ligand-binding domains. These data also suggest that the cross talk between SP- and SK-mediated responses may be explained by the homology of the extracellular structures between both receptor proteins, as well as the homologies of their peptide carboxyl termini.

These structural similarities between the SP and SK receptors and the fact that both receptors transduce their signals through similar G protein–coupled mechanisms are being used to isolate the gene for the SP receptor, using the polymerase chain reaction method and low-stringency hybridization techniques.

II. Solubilization and Characterization of Histamine Receptors.

Dr. Payan and Dr. M. Mitsuhashi previously showed that the cultured smooth muscle cell line DDT_1MF-2 expresses a large number (9.7×10^6 receptors/cell) of functional H_1-type receptors. The most recent work, using two different binding assays (gel filtration and polyethylene glycol precipitation), indicated that the [^{3}H]pyrilamine-binding activity was solubilized by 1% digitonin, with binding characteristics similar to those of intact cells. The solubilized proteins were then purified by sequential gel filtration, chromatofocusing, and reverse-phase high-pressure liquid chromatography. The calculated molecular weight of this purified pyrilamine-binding protein was 38–40 kDa on sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The binding of [^{3}H]pyrilamine to these 38–40 kDa proteins indicated a single class of binding site, with a K_d of 288 nM, which is equivalent to that of intact cells and digitonin-solubilized proteins. The computer analysis Scatfit also indicated that one molecule of [^{3}H]pyrilamine bound to one molecule of purified protein. Furthermore, a polyclonal antibody raised against the purified protein recognized the 38–40 kDa band by Western blotting techniques, specifically bound to the cell surface of DDT_1MF-2 cells, and inhibited [^{3}H]pyrilamine binding to these cells in a dose-dependent manner. These data strongly suggest that the purified 38–40 kDa protein is part of an antagonist-binding domain on the H_1 receptor on DDT_1MF-2 cells.

III. Multiple Signaling Pathways After Histamine Binding.

To analyze the complex activities of H_2-receptor activation on neutrophils, human HL-60 promyelocytic leukemia cells were differentiated by Drs. Payan and Mitsuhashi into neutrophils by incubation with dimethylsulfoxide, loaded with the Ca^{2+}-

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sensitive indicator dyes (indo-1 or Fura-2), and the levels of $[Ca^{2+}]_i$ measured in a fluorescent-activated cell sorter and fluorimeter, respectively. Histamine increased $[Ca^{2+}]_i$ in a dose-dependent manner, with a half-maximal concentration (EC_{50}) of $\sim 10^{-6}$–10^{-5} M, which exhibited H_2-receptor specificity. Prostaglandin E_2 and isoproterenol also induced $[Ca^{2+}]_i$ mobilization in HL-60 cells, whereas the cell permeable form of cAMP and forskolin failed to increase $[Ca^{2+}]_i$. Because H_2-receptor–mediated $[Ca^{2+}]_i$ mobilization was not inhibited by reducing the concentration of extracellular Ca^{2+} nor by the addition of Ca^{2+} channel antagonists, $LaCl_3$ and nifedipine, $[Ca^{2+}]_i$ mobilization is due to the release of Ca^{2+} from intracellular stores. Furthermore, both 10^{-4} M histamine and 10^{-6} M f-Met-Leu-Phe (fMLP) increased the levels of inositol triphosphate (1,4,5-IP_3). However, histamine-induced mobilization of $[Ca^{2+}]_i$ was inhibited by cholera toxin (CT) but not by pertussis toxin (PT), whereas the action of fMLP was inhibited by PT but not by CT. These data suggest that H_2 receptors on HL-60 cells are coupled to two different CT-sensitive G proteins and activate adenylate cyclase and phospholipase C simultaneously. Currently Dr. Payan and his colleagues are examining the G proteins that are associated with the H_2 receptor and whether they may be expressed at different times during cellular differentiation.

Dr. Payan is also Associate Professor in Residence in the Departments of Medicine (Infectious Disease) and Microbiology and Immunology at the University of California at San Francisco.

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SIGNAL TRANSDUCTION IN HEMATOPOIETIC CELLS

ROGER M. PERLMUTTER, M.D., PH.D., *Associate Investigator*

Dr. Perlmutter's research is focused on the developmental regulation of antibody repertoire diversification and on the mechanisms responsible for signal transduction in hematopoietic cells. During the past year, emphasis has been placed on the study of transgenic mice bearing altered signaling molecules. Disturbances in T cell function in such animals provide insight into normal lymphocyte signal transduction pathways.

I. Development of the Human Antibody Repertoire.

Antibodies are encoded by discontinuous germline gene segments that are juxtaposed through somatically propagated gene rearrangement events. Assembly of the adult antibody repertoire is developmentally programmed: a small number of germline antibody gene segments contribute preferentially to the generation of antibodies in fetal and neonatal B lymphocytes. Previous studies established that heavy-chain gene segments involved in these early rearrangements are concentrated near the 3′ end of the locus. Detailed analysis of the pattern of expression of antibody light chains in the human fetus revealed a hierarchical pattern of variable region gene segment rearrangement, indicating that the set of combining sites produced early in immune system ontogeny is limited. One curious feature of fetal antibody repertoires in general is the high frequency of autoreactive elements. This observation has led to the proposal that disease-inducing autoantibodies of the type encountered in rheumatologic illness result from inappropriate expansion of preexisting self-reactive B cell clones. At the same time, considerable evidence supports the view that most autoantibodies arise through antigen-driven somatic mutation. These alternative views of the pathogenesis of autoimmunity can now be productively addressed, using the large database of fetal antibody sequences developed in Dr. Perlmutter's laboratory and new microscale methods for determining the sequences of pathologic autoantibodies.

II. Protein Tyrosine Kinase Signaling Elements in Lymphocytes.

Although steady progress has been made in identifying the receptors on lymphocytes that permit recognition of foreign macromolecules, no consensus has emerged regarding the signaling process that permits antigen recognition to induce lymphocyte activation. Members of Dr. Perlmutter's laboratory have identified two membrane-associated protein tyrosine kinases ($p56^{lck}$ and $p59^{fynT}$) that are lymphocyte-specific signal transduction elements. Previous studies defined the *lck* gene by virtue of its overexpression in a murine lymphoma cell line and demonstrated that, like several other protein tyrosine kinases, the activity of $p56^{lck}$ is regulated by phosphorylation on a carboxyl-terminal tyrosine residue (Tyr505). Substitution of phenylalanine for tyrosine at this position generates a potent transforming element.

Two observations suggest that $p56^{lck}$ is intimately involved in T cell signal transduction. First, $p56^{lck}$ is physically associated with the CD4 and CD8 molecules that form part of the T cell antigen recognition complex. Second, T cell activation results in rapid conversion of $p56^{lck}$ to a p60 form and in the downregulation of the *lck* transcriptional unit.

To elucidate the function of $p56^{lck}$, Dr. Perlmutter and his colleagues are attempting to alter $p56^{lck}$ activity directly in otherwise normal T lymphocytes. Two distinct *lck* promoter elements that are separated by 35 kb of intervening sequence have been defined. Both elements behave in a lymphocyte-specific fashion in transgenic mice. The most 3′ of these promoter elements have been used to generate mice that bear an activated version of $p56^{lck}$ containing phenylalanine at position 505. The T cells from these animals have an altered T cell signaling profile, in that they can no longer be activated appropriately by mitogens. These observations focus attention on $p56^{lck}$ as a mediator of signals from the T cell antigen receptor complex.

In related studies, a second lymphocyte-specific protein tyrosine kinase has been identified. The product of alternative splicing of transcripts from the *fyn* gene, $p59^{fynT}$ contains an unusual catalytic domain structure, suggesting that the substrates for this kinase will differ from those phosphorylated by $p56^{lck}$. In addition, $p59^{fynT}$ does not interact with CD4 or CD8. Interestingly, $p59^{fynT}$ is greatly overexpressed in lymphocytes from *lpr/lpr* mice that suffer from a lymphoproliferative abnormality. This observation suggests that $p59^{fynT}$ will also assist in regulating T cell activation.

A third kinase under study is $p59^{bck}$, which, although present at low levels in B lymphocytes, is

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expressed primarily in myeloid cells, particularly granulocytes. As in the case of $p56^{lck}$, mutation of a carboxyl-terminal phosphorylation site unmasks the transforming ability of $p59^{hck}$. Since $p59^{hck}$ is maximally expressed in terminally differentiated cells, it almost certainly regulates aspects of myeloid cell function unrelated to replication. Preliminary studies suggest that $p59^{hck}$ is involved in the control of cytokine release. Current attempts to define the functions of $p56^{lck}$, $p59^{fynT}$, and $p59^{hck}$ involve the use of transgenic animals to alter the normal pattern of expression of these kinases and the use of retroviral vectors to introduce activated versions of these signaling molecules into experimentally manipulable cell lines.

III. The *lck* Promoter System as a Tool for Investigating T Cell Development.

Both promoters of the *lck* gene are lymphocyte-specific transcriptional regulatory elements. However, although the upstream promoter is active in essentially all T cells, the downstream promoter is expressed almost exclusively in thymocytes. These two regulatory elements function appropriately in transgenic animals and hence can be variously used to investigate features of normal T cell development. For example, four lines of animals bearing the downstream *lck* promoter coupled to SV40 large T antigen have been propagated for more than a year. These animals predictably develop thymomas with a latency of ~18 wk. Remarkably, cells from these thymic tumors can be readily propagated *in vitro* and in many cases appear to follow a normal differentiative program during continuous culture. Analysis of these cell lines may permit the identification of genes involved in the intrathymic maturation of T cells.

Dr. Perlmutter is also Associate Professor of Medicine and of Biochemistry at the University of Washington School of Medicine.

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REGULATION OF EXPRESSION OF CLASS II MAJOR HISTOCOMPATIBILITY COMPLEX AND HUMAN IMMUNODEFICIENCY VIRUS GENES

B. MATIJA PETERLIN, M.D., *Assistant Investigator*

Dr. Peterlin and his colleagues are studying the regulation of expression of genes that affect the immune system, namely those that code for class II major histocompatibility complex (MHC; class II, DR) antigens and human immunodeficiency virus (HIV) proteins. Different levels of expression of these proteins in humans lead to severe combined immunodeficiency and predispose to 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; the only hereditary deficiency in a regulatory protein in humans) and by HIV-encoded transactivators TAT and REV.

I. Regulation of Class II Gene Expression.

Class II antigens are peptide carriers that present self and foreign peptides to T cells to initiate and help T helper cells to interact with B cells to propagate the immune response. Developmentally, class II antigens must be expressed in the thymus to tolerize T cells to self proteins and to restrict T cells to nonself peptides. For these interactions to occur, the expression of class II genes must be carefully regulated.

A. *Cis-acting sequences.* Dr. Peterlin has extensively characterized transcriptional enhancer and promoter elements in the DRA gene. DRA promoter is bipartite, consisting of upstream promoter elements that are activated by interferon-γ and can confer B cell specificity and downstream promoter elements that position RNA polymerase II and lead to proper initiation of transcription. It is in these upstream promoter elements that DOB, DQA2, and DQB2 genes have mutations that result in aberrant expression (DOB) or nonexpression (DQA2, DQB2) of these genes. Furthermore, by using cluster point mutations in the DRA promoter, Dr. Peterlin found that identical cis-acting sequences are required for interferon-γ inducibility and B cell specificity.

B. *Trans-acting factors.* Dr. Peterlin has characterized proteins that interact with DRA transcriptional enhancer and promoter elements. In the transcriptional enhancer, OCT-1 and OCT-2 bind to the octamer box and C/EBP (core/enhancer binding protein) to the core enhancer element. In the promoter, OCT-1 and OCT-2 bind to the octamer, NF-Y to the Y box, NF-Xc and X2 to the X box, and NF-Zc and Z2 to the Z box. The Z box is a duplication of the X box, since in competition experiments, X box oligonucleotides compete off Z box–binding proteins. Proteins that interact with X and Z boxes are not fundamentally different in cells representing distinct class II phenotypes. Thus tissue specificity might be due to proteins that interact with these DNA-binding proteins. Dr. Goran Andersson in Dr. Peterlin's laboratory has now isolated four cDNA clones that code for proteins that bind to X and Z boxes. They have been extensively characterized; two code for NF-Xc and one for NF-X2.

C. *BLS II and class II–negative B cell lines.* Dr. Andrew Calman in Dr. Peterlin's laboratory developed class II–negative mutant B cell lines RM2 and RM3. Recently, other class II–negative B cell lines and BLS II cells were obtained. By transient fusions of these cells, three complementation groups leading to nonexpression of class II in B cells are found. None of these cells have gross defects in NF-Xc and NF-Zc. Although Dr. Peterlin has attempted to rescue these cells by gene complementation, using expression cDNA libraries, he has not yet rescued the defect in RM3. Genetic approaches and cDNA clones isolated by Dr. Andersson are being used to complement class II–negative and BLS II cells.

II. Regulation of HIV Gene Expression.

HIV causes acquired immune deficiency syndrome (AIDS) and requires the expression of the TAT and REV proteins for efficient viral replication and gene expression. Dr. Peterlin studies the mechanism of action of TAT on its trans-active-responsive region (TAR).

A. *Trans-activation by TAT.* The 15 kDa protein TAT is encoded by doubly spliced HIV RNA; TAT appears early in the viral life cycle and acts on 5′ HIV RNA sequences. Interactions between TAT and TAR lead to increased levels of HIV RNA and even higher levels of HIV proteins. Dr. Peterlin determined that TAT does not increase the rates of initiation of HIV transcription but facilitates expression of full-length HIV RNA. In the absence of TAT, short tran-

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scripts corresponding to the stem-loop in TAR are recovered. Quantitative differences in nuclear runons and recoveries of RNA can be interpreted as representing premature termination of transcription. Alternatively, TAT could prevent rapid degradation of nascent RNA in the nucleus. Research efforts have focused on exact requirements of cis-acting sequences in TAR and possible interactions between TAT and TAR. First, Dr. Peterlin determined that the stem-loop in TAR is required for trans-activation by TAT and has to be located very close to the site of initiation of transcription. Second, proteins that bind to TAR DNA are not required for trans-activation. Third, not only the structure of TAR RNA but also sequences near the loop of the stem-loop in TAR are very important. So far, no specific binding of TAT to TAR RNA has been observed. Moreover, trans-activation by TAT could not be reproduced in simple *in vivo* or *in vitro* systems, including *Escherichia coli*, yeast, *Xenopus* oocytes, and HeLa nuclear extracts.

B. *Activation of HIV gene expression.* In contrast to effects of TAT that occur after transcription has initiated, there are inducible trans-acting factors that are required for initiation of HIV transcription. These include NF-κB and NFAT-1 (nuclear factor of activated T cells), which bind to the transcriptional enhancer and upstream U3 sequences, respectively. Subtle differences between two isolated HIV viruses, HIV-1 and HIV-2, were noted in mechanisms of transcriptional activation. These include lower levels of activation of HIV-2 as compared with HIV-1 and the lack of a second NF-κB site in HIV-2, which might explain longer latency and attenuated clinical course observed with HIV-2. Cooperative and multiplicative interactions between activation of transcription and trans-activation by TAT were observed, implying once again that these mechanisms are distinct and separable.

In addition to studies on the activation of the HIV long terminal repeats (LTRs) by mitogens and T cell agonists, Dr. Peterlin investigated possible roles of other infections in the pathogenesis of AIDS. In collaboration with Ben Yen (University of California at San Francisco), he found that the X protein of hepatitis B, which is a known transcriptional activator, increases expression from the HIV-1 LTR in T cells. This activation occurs at the NF-κB sites and is independent of T cell activation. Thus hepatitis B virus could be an important cofactor in HIV disease progression. Additionally, in studies with Dr. Paul Luciw (University of California at Davis), Dr. Peterlin found that immediate early trans-activators of cytomegalovirus (CMV) also increase HIV-1–directed gene expression. In contrast to X protein, CMV trans-activators act on the HIV-1 TATA box.

Dr. Peterlin is also Associate Professor of Medicine and of Microbiology and Immunology at the University of California at San Francisco.

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T LYMPHOCYTE BIOLOGY AND GENETICS

ROBERT R. RICH, M.D., *Investigator*

Dr. Rich's laboratory studied three models of antigen recognition by T cells, focusing on structure-function relationships of major histocompatibility complex (MHC)-associated genes and gene products.

I. Maternally Transmitted Antigen (Mta).

Mta is a murine cell surface antigen of broad tissue distribution with features of a class I MHC molecule. It is identified by cytolytic T lymphocytes (CTL) and is unique in its maternal pattern of inheritance. One gene required for Mta expression, *Hmt*, has been mapped telomeric to the *Tla* region of the *H-2* complex and is thought to encode a class I polypeptide. Maternally inherited antigenic polymorphism, however, is determined by a mitochondrial gene, *Mtf*; two major genotypes, Mtf^{α} and Mtf^{β}, determine expression of the major antigenic phenotypes, Mta^{a} and Mta^{b}, respectively. Chloramphenicol, a specific inhibitor of mitochondrial translation, was employed to show that Mta expression reflected mitochondrial protein synthesis. Mta expression was also sensitive to treatment with monensin, which prevents protein movement through the Golgi apparatus. These data suggested that Mta expression requires a short-lived product of mitochondrial protein synthesis that may be transported through the Golgi apparatus to the cell surface.

More recent studies investigated the structural requirements for a specific amino-terminal sequence from the mitochondrial gene, ND1, as a possible candidate for the *Mtf* gene product. These studies, employing synthetic peptides added to culture media, confirmed the finding of Dr. Kirsten Fischer-Lindahl (HHMI, University of Texas Southwestern Medical Center at Dallas) that antigenic polymorphism of Mta^{a} versus Mta^{b} was determined by substitutions at position 6 of this highly hydrophobic peptide. The length of the peptide required for recognition by Mta-specific CTL clones ranged from 6 to 12 amino acids, with some clones recognizing an amino-terminal hexamer, whereas others required 8 or 12 amino acid peptides for optimal lysis by Mta-specific CTL. Formyl substitution was required on the amino-terminal methionine of the *Mtf* peptide. Mitochondrial proteins, in contrast to proteins encoded by the nuclear genome, are initiated by formyl-methionine, perhaps as a remnant of their presumed prokaryotic ancestry. Synthetic peptides initiated with nonsubstituted or acetylated methionine were not recognized by Mta-specific CTL. Inhibition studies suggested that the formyl group was required for effective interaction with the class I *Hmt* polypeptide in construction of the Mta antigen. These studies suggest possible novel functions of the *Hmt* gene product in transport of highly hydrophobic leader sequences of mitochondrial gene products and/or in host defenses to fMet-initiated (i.e., bacterial) antigens.

II. Structural and Functional Analysis of Human HLA Class II Genes and Antigens.

Previous studies from Dr. Rich's laboratory demonstrated that HLA class II–bearing cells from some $DR1^{+}$ subjects did not stimulate DR1-restricted, trinitrophenyl-specific $CD4^{+}$ T lymphocyte clones. Such cells were also unable to stimulate DR1-specific alloreactive T cell clones. In general, nonstimulators were of the extended haplotype *DR1,B14* and/or expressed the HLA-associated gene defect for nonclassical 21-hydroxylase deficiency. Analysis of DR molecules from nonstimulators led to identification of a class II MHC molecule with unique biochemical properties. Although immunoprecipitated with anti-DR monoclonal antibodies, it migrated under reducing conditions in two-dimensional gels as a single molecular species of ~50 kDa. Surprisingly, several $DR1^{+}$ individuals heterozygous for variant and wild-type DR1 genes displayed a nonstimulatory phenotype. Because an initial genomic analysis revealed a *Bgl*II restriction polymorphism in the 3′-untranslated region of the DRA gene that was invariably associated with the biochemical abnormality, cDNAs for the DRA and DRB genes were amplified, cloned, and sequenced. Analysis of the DRA gene from variant DR1 cell lines revealed only a silent nucleotide substitution in codon 77. In addition to a silent mutation in codon 78, the DRB gene had coding mutations in two adjacent codons, 85 and 86, in which GTT(Val) GGT(Gly) was replaced by GCT(Ala) GTG(Val). From studies from a molecular model of class II MHC proteins, these variant residues are hypothesized to lie at the end of the DRB α-helix thought to be involved in antigen binding. In addition to affecting T cell recognition, these mutations may alter the avidity of the DRA and DRB polypeptides for

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one another, resulting in the anomalous biochemical properties of the variant molecule.

III. Enterotoxin-mediated T Cell Activation.

Studies of the mechanisms of human T cell activation led to the observation that the *in vitro* proliferative response of purified T cells to *Staphylococcus* enterotoxin A (SEA) was dependent on the presence of HLA class II–bearing cells in the culture system. Class II–positive accessory cells (Raji and HLA-DR, -DQ, or -DP–transfected mouse fibroblasts) supported proliferative responses of T cells to SEA, while class II–negative versions of these cells did not. Unlike conventional antigens, the response to SEA was not restricted by the responder haplotype. These data suggested a novel interaction of SEA with class II molecules in formation of a mitogenic complex. This hypothesis was confirmed by demonstration that fluorescein-conjugated SEA bound to HLA class II–transfected mouse fibroblasts but not to untransfected cells. Moreover, by direct immunoprecipitation with SEA-Sepharose, bands were precipitated from Raji cells and class II transfectants that comigrated with the class II A and B chains precipitated with the class II–specific monoclonal antibody L243. In contrast, SEA-Sepharose did not precipitate any proteins from class II–negative cells. Such data led to the conclusions that SEA is an MHC–class II binding protein, that this binding is specific and of relatively high avidity, and that it is central to the activity of SEA as a T cell mitogen. Together with similar studies from other laboratories, as well as recent data indicating that the enterotoxins specifically stimulate T cell receptors bearing particular Vβ segments, these findings have provided insights into the extraordinary nature of this class of molecules and their possible involvement in bacterial host defenses and the pathogenesis of enterotoxin-mediated diseases.

Dr. Rich is also Professor of Microbiology and Immunology and of Medicine at Baylor College of Medicine and Attending Physician at its affiliated hospitals.

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LYMPHOCYTE SURFACE ANTIGENS IN HEALTH AND DISEASE

BENJAMIN D. SCHWARTZ, M.D., PH.D., *Investigator*

The major histocompatibility complex class II molecules are crucial to both normal immune responses and abnormal immune responses leading to the development of autoimmune disease. These molecules are highly polymorphic, and it is this polymorphism that is ultimately responsible for conferring both specific immune responsiveness and disease predisposition. The class II molecules consist of an α-chain glycoprotein and a β-chain glycoprotein; both are encoded by genes contained within the major histocompatibility complex. The proposed structure of the class II molecule suggests that the α1 domain of the α-chain and the β1 domain of the β-chain form a structure that consists of eight β strands forming a β pleated sheet, on which rest two α-helices that form a groove or cleft, which binds the antigenic peptide. This complex of the antigenic peptide and the class II molecule is the ligand that is recognized by the antigen-specific receptor on T lymphocytes.

The class II molecules have a limited tissue distribution and are expressed predominantly on immunocompetent cells, including B cells, antigen-presenting cells, and (in humans) activated T cells. However, class II expression can also be induced on a variety of other cells on which expression is normally absent. Dr. Schwartz and his colleagues have examined the regulation of class II gene expression, the binding of antigenic peptides by class II molecules, and the presentation of antigenic peptide to T cells.

I. HLA Class II Gene Regulation.

Last year Dr. Schwartz's laboratory reported the development of the Southwestern methodology to identify and clone the cDNAs that encode DNA-binding proteins. One such protein binds to a 48 bp oligomer, the X-Y box. The X-Y box corresponds to a portion of the class II gene promoter region that spans two highly conserved sequence motifs, the X box and Y box, and the spacer region between them, and has been shown to be essential for class II gene expression. The protein binding to the X-Y box, YB-1, was shown to be specific for the inverted CCAAT box sequence in the Y box.

Analysis of class II–positive and –negative cell lines indicated that although YB-1 is ubiquitous, YB-1 mRNA levels vary inversely with class II mRNA levels. This inverse correlation was supported by the observation that interferon-γ–induced class II expression on class II–negative U937 cells is accompanied by a fall in YB-1 levels. These results suggest that YB-1 is a repressor factor. This laboratory has also cloned and partially sequenced a murine homologue of YB-1 that is similar to human YB-1.

Because of a report that some CCAAT box–binding proteins are heterodimers, the possibility that YB-1 might be one subunit of a heterodimeric protein was explored. A λgt11 cDNA library prepared from the DR5 B lymphoblastoid cell line Swei was probed with the Y box to which the YB-1 protein had been bound. A second cDNA was isolated and shown to encode a protein that binds to the YB-1–Y box complex. In the absence of YB-1, this protein, YB-2, did not bind to the Y box itself but did bind weakly to the X-Y box. In the presence of YB-1, YB-2 bound strongly to both the X-Y box and the Y box. Levels of YB-2 mRNA also inversely correlated with class II mRNA levels. Restriction mapping, Southern analysis, and DNA sequencing confirmed that YB-2 was distinct from YB-1. Gel retardation studies in which the interaction of YB-1 and YB-2 was studied suggested that YB-2 interacts with YB-1 to prevent it from binding to the X-Y box.

A study of the proteins within various nuclear extracts that interact with the class II gene promoter regions has also been initiated. Gel retardation analysis of DNA-binding proteins within these nuclear extracts suggests that the binding patterns observed are similar in class II–negative and class II–positive cells. In addition, class II–negative cells appear to have higher levels of DNA-binding proteins and/or higher affinity binding proteins than do class II–positive cells. These results are reminiscent of the higher levels of YB-1 and YB-2 mRNAs observed in class II–negative cells compared with class II–positive cells and support the notion that, in the class II system, high levels of certain transacting factors act to repress class II transcription.

II. Interaction of Class II Molecules and Antigenic Peptides.

In collaboration with Drs. Immanuel Luescher, Dan Crimmins, and Emil Unanue, Dr. Schwartz has been studying the interaction of class II molecules and antigenic peptides. In the mouse system, the I-A^k molecule is known to bind the hen egg white lysozyme peptide (HEL) 46–61. Conjugates of this

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peptide with a radiolabeled iodinated photoaffinity probe were synthesized and, when bound to I-A^k, were capable of stimulating an HEL-specific T cell hybridoma. This suggests that the photoaffinity conjugate binds I-A^k in a manner similar to the unmodified peptide. The sites on the I-A^k molecule that were photoaffinity-labeled with iodo, 4-azido salicyloyl–HEL 46–61, were identified. Protease V8, trypsin, and endoproteases Arg-C and Lys-C digestions were performed on photoaffinity-labeled I-A^k that had been biosynthetically labeled with [^{35}S]cysteine, [^{3}H]tryptophan, or [^{3}H]proline. The peptide maps suggested that the photoaffinity probe labeled a hydrophobic site that is formed by sequences in the first halves of the second domains of the α- and β-chains and is located in the vicinity of the allele-specific antigen-binding site. These results were confirmed by sequencing of the [^{3}H]proline-labeled class II molecules. These results suggest that HEL 46–61 binds to the antigen-binding site and that the photoreactive group extends beyond the peptide-binding cleft and is able to interact with a hydrophobic site on the I-A^k molecule that is in the immediate vicinity.

III. T Cell Recognition of the Class II–Antigenic Peptide Complex.

In collaboration with Drs. Lawrence Brown, Vivian Braciale, Neal Nygard, and Tom Braciale, Dr. Schwartz has examined the recognition of the class II–antigenic peptide complex. The antigenic site on the influenza hemagglutinin (HA) molecule that is recognized by a human influenza-specific cytotoxic T cell clone was identified, and the human class II molecule that interacts with this portion of the HA molecule was determined. T cell clone V1 was demonstrated to recognize synthetic peptides of the HA sequence from influenza strain A/JAP/57 corresponding to the region spanning residues valine 129 to glutamine 140 when presented in the context of the HLA-DR11 subtype of HLA-DR5. The residues within the synthetic peptide that contribute to the binding to DR11 were identified, as were the residues that appear to interact with the T cell receptor.

Photoaffinity probe conjugates of the HA 129–140 peptide demonstrated that iodinated photoconjugates would bind to L cell transfectants bearing DR11 but not to transfectants bearing other class II molecules encoded by the DR11 haplotype. The sites on the DR11 molecule that interact with the photoaffinity conjugate are presently being determined.

Dr. Schwartz is also Professor of Medicine and of Molecular Microbiology at Washington University School of Medicine, Physician at Jewish Hospital, St. Louis, and Associate Physician at Barnes Hospital, St.Louis.

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MOLECULAR ASPECTS OF DIFFERENTIATION AND PROLIFERATION OF THE LYMPHOID SYSTEM

CRAIG B. THOMPSON, M.D., *Assistant Investigator*

Dr. Thompson's research is focused on the molecular events associated with cellular differentiation and proliferation of the immune system. Over the preceding year the laboratory has concentrated on two major projects: 1) characterizing B cell development in the avian bursa of Fabricius and 2) defining molecular mechanisms involved in regulating gene expression during the activation of normal human T cells.

I. Bursa of Fabricius as a Model System for the Study of Lymphoid Development.

The bursa of Fabricius provides a unique organ for understanding lineage-specific development in a multicellular organism. B cells in the chicken, unlike in mammals, develop in a single wave of differentiation, beginning with commitment of progenitor cells to B cell differentiation between days 10 and 15 of embryogenesis. By day 18 of embryogenesis, all lymphoid progenitor cells capable of differentiation along the B cell lineage have migrated to the bursa of Fabricius. After migration to the bursa, these lymphoid progenitors enter exponential growth and begin to populate each of the 10^4 bursal follicles. Between day 18 of embryogenesis and 2–4 weeks of age, B cells undergo a stage of bursal-dependent differentiation. By the end of this period, chickens are able to mount primary immune responses against virtually all antigens. In addition, by 4 weeks of age sufficient numbers of B cells have migrated from the bursa to peripheral lymphoid organs so that the B cell immune system can be maintained, even if the bird is bursectomized. Bursectomy of chicks after 4 weeks of age has no long-term effect on the development and maintenance of the B cell immune system in adult birds.

Because of the central nature of the surface Ig molecule to B cell development in mammals, Dr. Thompson and his colleagues have undertaken a characterization of the chicken Ig light-chain (Ig_L) locus during bursal development. Several years ago, this locus was shown to have only one V region capable of rearrangement. To create an immunological repertoire, chickens must diversify the coding sequence of this functional V gene segment at some point during development. This diversification occurs subsequent to Ig_L rearrangement during the bursal-dependent phase of B cell development. Ig_L gene diversification is limited to the rearranged V gene segment and occurs by a gene conversion mechanism, using V region pseudogenes as sequence donors. In contrast to diversification, rearrangement of the Ig_L gene is not dependent on the bursal environment. B cell progenitors rearrange their Ig_L gene between days 10 and 15 of embryogenesis, prior to migration to the bursa. Ig_L gene rearrangement occurs by a deletional mechanism in which a precise joining of the Ig_L recombination signal sequences leads to a circular episomal element. During this deletion it appears that single nonrandom bases are added to both the V and J coding segments. Subsequent V-J joining appears to occur at random. Most progenitor B cells appear to rearrange only a single Ig_L allele. The high frequency of in-frame alleles observed in avian B cell lines appears to result from the selective amplification of cells with productive Ig_L rearrangements during bursal development, between days 12 and 18 of embryogenesis. The continued characterization of B cell differentiation within the bursa of Fabricius remains a central focus of Dr. Thompson's laboratory.

II. Normal Human T Cells as a Model System for the Study of Gene Expression During Cellular Activation.

Nuclear proto-oncogenes have been characterized as having a potential role in the regulation of cellular proliferation. This has led to an expanded interest in the molecular events associated with the transition of a lymphocyte from a quiescent state to one of either cellular proliferation or effector function. To study these events in a normal quiescent cell population, Dr. Thompson and his colleagues have concentrated on characterizing molecular events associated with human T cell activation. Although many other laboratories have a similar interest, Dr. Thompson's laboratory is using defined purified populations of normal resting human T cells isolated by lymphopheresis and negative selection using monoclonal antibodies. The laboratory has concentrated on how molecular events transduced through the T cell receptor and accessory T cell surface molecules regulate the expression of a variety of genes associated with cellular proliferation and T cell function. These genes have included the nuclear proto-oncogenes, lymphokine genes, T cell receptor genes, and activational T cell surface markers.

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These studies have led to the characterization of physiologic roles for transcriptional attenuation and mRNA stability in the regulation of specific T cell genes. In addition, an apparently novel T cell activation pathway has been characterized that is both defined and regulated by the CD28 surface molecule. This pathway was originally defined because CD28 activation was found to render T cell proliferation resistant to the immunosuppressant cyclosporine. Further studies have demonstrated that the CD28 pathway coordinately regulates the expression of a group of lymphokine genes in antigen-activated T cells. The lymphokines that appear to be regulated by this pathway include interleukin-2, interferon-γ, lymphotoxin, tumor necrosis factor-α, and granulocyte macrophage colony-stimulating factor. These lymphokines have previously been described as being associated with helper T cell–induced delayed-type hypersensitivity reactions. The CD28 activation pathway enhances the expression of this class of lymphokine genes, as a result of a specific increase in the stability of lymphokine mRNAs. The laboratory is continuing its work on 1) characterizing the molecular basis for the stabilization of lymphokine genes by the CD28 activation pathway and 2) defining the role of transcriptional attenuation in regulating T cell gene expression through defined surface activation pathways.

Dr. Thompson is also Assistant 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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DEVELOPMENT OF γδ T CELLS

SUSUMU TONEGAWA, PH.D., *Investigator*

Dr. Tonegawa's major research interest continues to be the newly discovered T cell receptor (TCR) γδ and T cells bearing this TCR. Because nothing was known about this type of TCR and T cells when the TCR γ gene was discovered in Dr. Tonegawa's laboratory in 1984, the research has been progressing in a reverse direction—from the gene to the protein, from the protein to the cell, and from the cell to the function. The function of γδ T cells is still unknown. However, much information has been learned during the past year about the diversity, tissue distribution, development, and specificity of these T cells, and some plausible roles for these T cells are emerging.

I. Preparing Monoclonal Antibodies Reactive with Mouse γδ TCR.

Monoclonal antibodies directed against the native receptor are useful in the study of the nature of γδ TCR and the role of γδ T cells. By immunizing Armenian hamsters with the γδ TCR–CD3 complex partially purified from a lysate of a γδ T hybridoma, KN6, Dr. Tonegawa's laboratory prepared three anti–γδ TCR monoclonal antibodies: 3A10, specific for a Cγ constant region determinant; 8D6, specific for $V_{\gamma 4}$- and $V_{\delta 5}$-encoded γδ TCR; and 5C10, specific for a KN6 TCR idiotope.

II. Distribution of γδ T Cells.

The availability of the staining anti–γδ monoclonal antibody allowed Dr. Tonegawa's laboratory to enumerate γδ T cells in developing thymus and peripheral lymphoid organs by flow cytometry and immunohistochemistry. As the earlier immunoprecipitation and molecular genetic analyses suggested, γδ T cells appear one to two days earlier (day 14.5 of gestation) in the fetal thymus than αβ T cells, but compose no more than a few percent of the total thymocytes or splenic T cells throughout the animal's life. Most of these T cells do not express CD4 or CD8. By contrast, following the reports of the occurrence of γδ T cells in epidermis and gut epithelia, Dr. Tonegawa's laboratory demonstrated that many organs carry γδ T cells that are frequently in association with their epithelial cells. These organs include large intestine, tongue, stomach, uterus, and vagina. Although some αβ T cells are also present in association with the epithelia of some of these organs, the majority of $CD3^+$ intraepithelial lymphocytes are clearly γδ T cells, suggesting a role for these T cells in the surveillance of epithelial cells. Dr. Tonegawa's laboratory refers to these γδ T cells as intraepithelial lymphocytes (IEL); the initial of each organ is used to designate various IEL subpopulations, such as i-IEL, s-IEL, r-IEL, and t-IEL for the IEL in intestine, skin, reproductive organs, and tongue, respectively.

III. γδ T Cell Subpopulations with Virtually No TCR and Abundant TCR Diversity.

The γδ T cells are composed of several different subpopulations, which are characterized by gene segments utilized to encode their TCR, by widely different degrees of TCR variability, by preferred localization in specific anatomical sites, and by the timing of their appearance in the developing thymus. Thus, following the demonstration that virtually all s-IEL γδ TCR are encoded by $V_5J_1C_1\gamma$ and $V_1D_2J_2\delta$ genes with no sequence diversity, Dr. Tonegawa's laboratory demonstrated that the TCR of virtually all r-IEL and t-IEL are encoded by $V_6J_1C_1\gamma$ with no sequence diversity and by the δ gene whose nucleotide sequence is identical to that of the δ gene encoding the s-IEL δ-chain. Furthermore, Dr. Tonegawa's laboratory demonstrated that most γδ thymocytes that appear early in fetal development (i.e., day 14–16 of gestation) and disappear thereafter express the same, undiversified TCR as that expressed on s-IEL. They also demonstrated that this wave of γδ thymocytes is followed by another wave that peaks around birth and is composed of T cells bearing the same, undiversified γδ TCR as that present on most r-IEL and t-IEL. In contrast, the TCR expressed on adult γδ thymocytes and on the γδ T cells of adult lymphoid organs are diverse, exhibiting both combinatorial and junctional diversity. The diversity of the i-IEL γδ TCR, on the other hand, seems to be intermediate, because the preferential utilization of $V_7J_1C_1\gamma$ genes virtually precludes the combinatorial diversity of their γ-chains.

IV. Silencer Model of αβ and γδ T Cell Development.

The discovery of γδ T cells raises the issue of their developmental relationship with αβ T cells. It

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was previously suggested that αβ T cells are generated only from those cells that failed to rearrange γ or δ genes productively. Dr. Tonegawa's laboratory tested this model by analyzing transgenic mice constructed with productively rearranged TCR γ and δ genes. Contrary to the prediction of the previously published model, neither the absolute number nor the proportion of αβ T cells is significantly altered in the thymus and spleen of the transgenic mice. In these αβ T cells the transgene γ is repressed, as are the endogenously derived C_1-associated γ genes present in many αβ T cells of normal (nontransgenic) mice.

The repression seems to be mediated by a C_1-associated "silencer" element active in αβ T cells, because a C_1-associated γ gene with limited flanking sequences introduced into αβ T cells, as opposed to the coexisting endogenous γ gene, is derepressed. Based on these results, Dr. Tonegawa and his colleagues argue that the failure to rearrange both γ and δ genes productively is not a requirement for the generation of αβ thymocytes. They propose that the putative machinery acting on the γ silencer is activated in a fraction of immature thymocytes and that it is from these cells that αβ thymocytes are generated. The silencer model of T cell development is supported by another observation: in transgenic mice constructed with the silencerless γ and δ genes, development of αβ T cells is severely blocked.

V. Recognition of a Self Major Histocompatibility Complex TL Region Product by γδ TCR.

To understand the function of γδ T cells it is essential to identify the ligand of the TCR. For this purpose Dr. Tonegawa's laboratory prepared γδ T hybridomas and used a growth inhibition assay to screen them for their specificity. This led to the identification of one hybridoma, KN6, specific to a product of the self major histocompatibility complex (MHC) TL region. The ligand appears to be present on thymocytes, splenocytes, peritoneal exudate cells, and embryonal carcinoma cell line PCC3. From the PCC3 cell, Dr. Tonegawa's laboratory cloned an MHC class I gene; the nucleotide sequence of this gene was different from that of any of the previously reported class I genes. This gene encodes the KN6 ligand, as demonstrated by the specific reactivity of L cell transfectants with the KN6 cells.

VI. Possible Functions of γδ T Cells.

Dr. Tonegawa's laboratory believes that the ligands of γδ TCR, like those of αβ TCR, are made up of an antigen-derived peptide complexed with a restricting element. It is suspected that generally the restricting elements for the γδ T cells are MHC class I or MHC class I–like molecules, such as those mapped in the TL region, which are distinct from those for $CD8^+$ αβ T cells. These class I molecules may have evolved to present a special set of antigens to the immune system, which might be explained by postulating a special intracellular pathway of peptide loading and/or common structural features of the presented peptides. Recent experiments have shown the recognition of mycobacterial heat-shock-like proteins by some γδ T cells; this suggests which set of proteins may be presented efficiently by the restricting elements for γδ T cells. It may be that γδ T cell subpopulations with diverse TCR are primarily directed to a variety of mycobacteria and parasitical protozoa known to produce constitutively distinct heat-shock-like proteins that are structurally related. The γδ T cell subpopulations with undiversified TCR then may recognize a host's own stress protein, which may be induced in the epithelial cells by a variety of unfavorable stimuli, such as viral infections, toxic chemicals, radiation, heat shock, and malignancy.

Dr. Tonegawa is also Professor of Biology at the Massachusetts Institute of Technology, Center for Cancer Research.

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STRUCTURE AND FUNCTION OF THE T CELL ANTIGEN RECEPTOR

ARTHUR WEISS, M.D., PH.D., *Associate Investigator*

Dr. Weiss's goal is to understand how T lymphocyte surface molecules regulate cellular responses.

The T cell antigen receptor (TCR) is among the many cell surface molecules that regulate T cell activation. The TCR is a remarkably complex seven-chain structure that consists of two subunits. 1) The ligand-binding subunit that is responsible for antigen recognition comprises the α/β-chain heterodimer (Ti). The α- and β-chains are clonally distributed and are derived from rearranging genes. 2) The CD3 complex, comprising five invariant chains, δ, ε, γ, and ζ_2. CD3 is thought to play a role in signal transduction.

The TCR activates two signal transduction pathways: the phosphatidylinositol (PI) and tyrosine kinase pathways. In the case of the PI pathway, stimulation of the TCR results in increases in inositol phosphates and diacylglycerol as a consequence of phospholipase C–induced hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2). PIP_2-derived second messengers, inositol 1,4,5-trisphosphate and diacylglycerol, are responsible for the observed mobilization in cytoplasmic free calcium ($[Ca^{2+}]_i$) and activation of protein kinase C. Several late biological responses by T cells are associated with these intracellular changes, including transcription of lymphokine and lymphokine receptor genes, expression of lymphocyte activation antigens, and activation of the cytolytic mechanism. The precise role of the components of the TCR and the identity of the intracellular elements involved in activation of the PI pathway are not understood.

The second signal transduction pathway regulated by the TCR involves the activation of a tyrosine kinase. This is best manifested by the appearance of several new tyrosine-containing phosphoproteins after TCR stimulation. Most notable is the tyrosine phosphorylation of the ζ-chain of the TCR. The kinase responsible for this activity is not intrinsic to the TCR itself and has not been identified. The relationship between this pathway and the PI pathway, as well as its role in cell activation responses, is not known.

I. Isolation of TCR Signal Transduction Mutants.

The complex nature of the TCR structure is not readily amenable to a simple structural and functional analysis. To identify the functional domains of the TCR and other components of the TCR-regulated PI signal transduction pathway, a somatic cell genetic system was developed to isolate TCR signal transduction mutants. A protocol involving ligand-induced growth inhibition and fluorescence cell sorting for cells that fail to increase $[Ca^{2+}]_i$ was used to isolate a family of three signal transduction mutants (J.CaM1–3) from the T cell leukemic line Jurkat. All three cells express high levels of TCR on the cell surface but fail to mobilize $[Ca^{2+}]_i$ or increase inositol phosphate metabolites in response to anti-Ti monoclonal antibodies. Two of the mutants, J.CaM1 and J.CaM3, do respond partially to a subgroup of anti-CD3 monoclonal antibodies. The similar functional phenotypes of these two mutants suggest that the molecules that are defective in these cells are involved in a similar function within a multimolecular complex. Utilizing a novel heterokaryon complementation assay, the defects in these cells have been mapped to three distinct genes other than the Ti chains. Current efforts are aimed at examining the functional competency of the tyrosine kinase pathway and at developing a genetic reconstitution system to identify the defective components within these cells.

Characterization of the responses of these mutants has also established that 1) ligand-induced TCR internalization is not dependent on TCR induction of the PI pathway; 2) all three mutants with distinct defects fail to produce interleukin-2 (IL-2), supporting a role for the PI pathway in this cellular activation response; 3) sustained activation of the PI pathway is required for commitment to IL-2 production; and 4) there is a directional transfer of information regarding ligand occupancy from Ti to CD3.

II. Function of the Human Muscarinic Receptor in T Cells.

As a further means of characterizing these mutants and to assess the role of the inositol phospholipid pathway in regulating the activation of T cells, a heterologous receptor that activates the inositol phospholipid pathway was expressed in Jurkat cells and the signal transduction mutants. The human muscarinic acetylcholine receptor subtype 1 (HM1) is expressed in neuronal, cardiac, and smooth muscle cells and functionally activates the PI pathway in these cells. When transfected into Jurkat cells, the HM1 receptor efficiently activates

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the PI pathway in response to carbachol, independent of the TCR. Moreover, stimulation of HM1 leads to several parameters of T cell activation, including IL-2 production, IL-2 receptor expression, and the expression of the T cell activation antigen CD69. Signal transduction events mediated by HM1 can even synergize with signals delivered through other T cell accessory molecules, such as CD28, in inducing IL-2 production. Finally, HM1 does not activate tyrosine kinases independent of one that is linked to the PI pathway. This suggests that the TCR induction of the PI pathway is sufficient for the activation of T cells.

The ability of the HM1 receptor to activate the PI pathway in Jurkat cells suggested that it would be a useful probe to analyze the functional competency of distal components of the PI signal transduction pathway in the J.CaM1–3 mutants. HM1 could activate the PI pathway in all three of these signal transduction mutants. This suggests that the defects in these cells affect components that are specific for the pathway activated by the TCR.

III. Identification of Ligand-induced TCR-associated Proteins.

In recent studies, two cell surface glycoproteins of 34 and 38 kDa have been identified that associate with the TCR after it has bound an agonist. Alkylation of the complex of the TCR and gp34 and gp38 is required to preserve their association. Association of these two glycoproteins with the TCR is a specific response that depends on stimulation of the TCR but not other cell surface molecules. The functional significance of their association is suggested by the fact that these proteins do not associate with the TCR on the signal transduction mutants J.CaM1 or J.CaM3 but do associate with the TCR of J.CaM2, despite its failure to mediate signal transduction through the PI pathway. Hence the association of gp34 and gp38 is not a consequence of signal transduction. Further characterization of gp34 and gp38 on normal T cells as well as on these mutants will help elucidate their role in TCR-mediated signal transduction.

The activation of T cells by antigen is a complex process that is initiated during a complex cell-cell interaction. These investigations are an attempt to define the role of one of the molecules, the TCR, primarily regulating these events. Such studies may not only help define the role of the TCR in T cell activation but may paint a broader picture of how cell surface receptors function in mediating transmembrane signaling events and in regulating cell interactions.

Dr. Weiss is also Associate Professor of Medicine and of Microbiology and Immunology at the University of California at San Francisco.

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NORMAL AND ABNORMAL LYMPHOCYTE GROWTH REGULATION

OWEN N. WITTE, M.D., *Investigator*

An understanding of the mechanisms used to regulate the normal production of lymphocytes in the bone marrow and thymus is necessary for the study of hypoproliferative states such as immunodeficiencies, as well as hyperproliferative states such as leukemias and lymphomas. Dr. Witte's laboratory has concentrated on the establishment of *in vitro* culture models to evaluate B cell production from primitive stem and progenitor elements and the growth deregulation caused by the expression of oncogenes that play a central role in the development of specific human leukemias.

I. Expression of the *BCR/ABL* Gene Family in Philadelphia Chromosome–Positive Leukemias.

Previous work has detailed the molecular events by which the Philadelphia chromosome translocation (t9:22), found in human chronic myelogenous leukemia and some cases of acute lymphocytic leukemia, creates related chimeric mRNAs encoding the P210 or P185 *BCR/ABL* oncogenes. The transcriptional regulatory apparatus and 5′-untranslated region of the *BCR* gene controls the expression of the gene, and the tyrosine kinase domain of the *ABL* segment is crucial for transforming activity. In collaboration with Dr. Christopher T. Denny (University of California at Los Angeles), the promotor segment of *BCR* has been isolated, sequenced, and functionally analyzed. It lacks a TATA box element and appears to be a GC-rich sequence with multiple SP1-binding sites that efficiently drives expression of heterologous test genes in a wide variety of cells.

Early lymphoid and myeloid cells are directly transformed by cDNA copies of *BCR/ABL* mRNAs introduced into hematopoietic culture systems *in vitro*. A number of surprising observations have been made with this system. First, recent studies have documented that the 5′-untranslated sequences, which are exceptionally GC rich, are a major determinant of pathogenesis. Only constructs with long untranslated regions (>400 bases) efficiently transform murine hematopoietic cells *in vitro* or *in vivo*. Second, the potency of transformation in all the *in vitro* and *in vivo* model systems evaluated correlates directly to the specific activity of the tyrosine kinase. The P185 gene product is more potent biologically than P210 in fibroblast and hematopoietic transformation models, and this is reflected in a 5- to 10-fold increase in specific tyrosine kinase activity measured on enzyme purified from mammalian cells or hyperexpressed from a baculovirus vector in insect cells.

The unique chimeric junctions of the *BCR/ABL* mRNAs were exploited to develop a sensitive polymerase chain reaction (PCR) diagnostic test to detect small numbers of Philadelphia chromosome–positive cells in clinical samples. This test has been used extensively in multiple centers to study the pathobiology of this group of leukemias. Recent observations on a group of chronic myelogenous leukemia patients treated by bone marrow transplantation have indicated that PCR analysis over the first six months post-transplant may be a useful indicator of cytogenetic, and eventually clinical, relapse.

II. Development of Selective Culture Conditions for Growth of Clonal Populations of B Lymphoid Progenitor Cells.

The natural history of chronic myelogenous leukemia and acute lymphocytic leukemia support the hypothesis that primitive stem cells are the primary target of the *BCR/ABL* oncogene. A major goal of Dr. Witte's laboratory has been to combine the action of specific oncogenes and selective *in vitro* growth conditions to isolate defined stages of hematopoietic development, including such stem cells. Previous attempts to combine long-term culture techniques selective for early B lineage precursors and pre–B cells with infection by the *BCR/ABL* oncogene resulted in overgrowth by pre–B cell types with immunoglobulin μ heavy-chain segments already rearranged to the DJ or VDJ configuration.

By modifying the specific culture conditions, including the use of clonally derived stromal cell feeder layers and oncogene expression constructs that minimize toxicity and maximize transformation activity, Dr. Witte and his colleagues have been able to isolate clonal lines of B lymphoid progenitor cells routinely. These continuous cell lines retain immunoglobulin μ heavy-chain genes in the germline configuration but can subsequently rearrange these genes and express immunoglobulin. Most importantly, although they contain and are growth-stimulated by a potent oncogene, these lines are nontumorigenic on transplantation into SCID (severe combined immunodeficient) mice and effec-

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tively reconstitute B lineage function in such mice, as exemplified by production of serum IgM. Co-inoculation of T cells with the cultured B cell progenitor lines induced class switching and the expression of multiple immunoglobulin isotypes. These studies help to define the earliest known cell type for the B cell lineage and should be valuable for the definition of developmental controls for the B cell lineage and the relationship of B cell progenitors to more primitive stem cells.

Dr. Witte is also Professor of Microbiology and Member of the Molecular Biology Institute at the University of California at Los Angeles.

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IV. NEUROSCIENCE

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IV. PROGRAM IN NEUROSCIENCE

The Institute's Program in Neuroscience was initiated in 1984. Investigators in this area are working at The Johns Hopkins University; the University of California at Berkeley, at San Diego, and at San Francisco; the Massachusetts General Hospital; the Massachusetts Institute of Technology; the University of Texas Southwestern Medical Center at Dallas; Yale University; Columbia University; the University of Pennsylvania; The Salk Institute for Biological Studies; the University of Washington; the State University of New York at Stony Brook; and Brandeis University. Among the topics under investigation are the development of the nervous system, the molecular basis of neurotransmission, the structure and function of receptors for neurotransmitters, the mechanisms responsible for long-term changes in the nervous system, and the cellular and molecular basis of certain neurological diseases.

Senior Investigator Eric R. Kandel, M.D. (Columbia University) and his colleagues have continued to study elementary forms of learning and the relationship between short- and long-term memory. During the past year they have examined three issues: 1) some of the molecular mechanisms of long-term memory, 2) the interactions between presynaptic neurons and target cells that lead to neuronal growth changes associated with long-term memory, and 3) cloning of potassium channels in *Aplysia* based on their homology to the *Drosophila Shaker* locus.

Changes in synaptic effectiveness are believed to underlie all memory and learning. These changes are produced by modulating neural inputs to specific nerve cells that operate through receptor-mediated processes or signal transduction mechanisms. Each of these transduction mechanisms can change the biochemical properties of the target nerve cell through the generation of a second messenger that, in turn, activates secondary effectors such as protein kinases. Investigator James H. Schwartz, M.D., Ph.D. (Columbia University) and his co-workers have examined the mechanisms by which these enzymes are altered in identified *Aplysia* neurons that have been shown to mediate a simple form of learning and how these kinases are made to act long after the initial second messenger has been dissipated. The laboratory has discovered mechanisms for keeping the cAMP-dependent protein kinase and protein kinase C persistently active. These persistence mechanisms are important, because they may provide for part of the molecular basis of learning and memory.

Neurons communicate with one another at synapses, which are specialized points of contact between the processes of one neuron and the cell body or dendrites of another. The presynaptic neuron transmits a signal by releasing one or more packets of a specific chemical, a neurotransmitter, from the ends of its processes; the neurotransmitter in turn is detected by specialized receptors on the surface of the postsynaptic or receiving neuron. This method of transmission is somewhat uncertain, however, because the release of the packets of neurotransmitter is a stochastic or random process. To understand neural signaling, then, the probabilistic laws that govern neurotransmitter release must be elucidated. Technical difficulties have, however, prevented these laws from being thoroughly investigated in synapses in the brain. By exploiting some of the advantages of neural circuits that can be formed by neurons growing in cell culture dishes, the laboratory of Investigator Charles F. Stevens, M.D., Ph.D. (Yale University) has been able to characterize this probabilistic release process that underlies neural signaling for one important class of synapse.

In neurons the storage and secretion of neurotransmitters is mediated by specialized cellular organelles called synaptic vesicles. The recent investigations of Associate Investigator Thomas C. Südhof, M.D. (University of Texas Southwestern Medical Center at Dallas) have focused on the composition of the membranes of such vesicles. Using molecular cloning techniques, he and his colleagues have succeeded in elucidating the primary structures of several vesicle membrane proteins and are providing tools to study the functions of these proteins in the biogenesis and exocytosis of synaptic vesicles. Their work is relevant not only for an understanding of vesicle biogenesis and targeting in neurons but also for the elucidation of the ubiquitous recycling of membrane that occurs in all secretory cells.

The research in the laboratory of Associate Investigator Richard L. Huganir, Ph.D. (The Johns Hopkins University) is directed toward understanding the molecular mechanisms that underlie the modulation of synaptic function. Dr. Huganir's laboratory is using the nicotinic acetylcholine receptor, which is by far the best characterized neurotransmitter receptor, as a model system to study the role of pro-

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tein phosphorylation in the regulation of neurotransmitter receptor and ion channel function. His group has found that the nicotinic receptor is phosphorylated at seven distinct 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 different neurotransmitters, suggesting that one consequence of the co-release of transmitters is that it provides for the regulation of the sensitivity of receptors and hence modulates synaptic transmission.

Ion channels in plasma membranes determine the electrical behavior of such excitable cells as muscles and neurons, and in all cells they control the entry and egress of small ions. The laboratory of Assistant Investigator Gary Yellen, Ph.D. (The Johns Hopkins University) is concerned with the molecular mechanisms that determine the ion selectivity and gating control of ion channels. By using genetic techniques to discover and manipulate the structure of ion channel proteins, it is possible to correlate particular parts of the protein with particular functions. The well-characterized nicotinic acetylcholine receptor channel is used as a starting point. This channel produces electrical excitation in nerve and muscle cells. The laboratory is employing several approaches to study mutations of the structure of this channel and is attempting to clone a gene for a closely related but inhibitory channel protein.

Research in the laboratory of Assistant Investigator Susan G. Amara, Ph.D. (Yale University) is concerned with the molecular biology and regulation of genes encoding certain neurotransmitter transporters that have a central role in synaptic transmission and are the site of action for a wide range of clinically important drugs. Most recently the group has pursued a molecular characterization of the proteins responsible for neurotransmitter reuptake and has developed a functional expression system for identifying the genes encoding these carriers. They also have continued to explore the basic mechanisms for regulating both neuronal gene transcription and alternative pathways of pre-mRNA processing, using a neuropeptide gene family as a model system.

The research of Associate Investigator Steven A. Siegelbaum, Ph.D. (Columbia University) and his colleagues is concerned with the modulation of the electrical activity of nerve cells by neurotransmitters. During the past year they have defined the molecular mechanism by which a particular neuropeptide inhibits the activity of a sensory neuron in the marine snail *Aplysia californica*. They have shown that a membrane fatty acid, arachidonic acid, is metabolized into 12-HPETE (12-hydroperoxyeicosatetraenoic acid), which then acts directly to open a potassium channel in the sensory neuron membrane, leading to an inhibition of electrical excitability. This group has also shown that arachidonic acid can modulate ion channel activity in vertebrate sympathetic neurons. Here arachidonic acid acts to block an excitatory calcium channel, leading to an inhibition of neuronal activity.

Investigator Richard Axel, M.D. (Columbia University) and his colleagues have been studying the molecular basis for the diverse physiologic actions of the neurotransmitter serotonin (5HT). By combining the techniques of molecular biology with those of electrophysiology, his laboratory, in collaboration with that of Dr. Thomas M. Jessell (HHMI, Columbia University), has isolated and characterized the genes encoding two distinct 5HT receptors. They have demonstrated that these genes define a new family of neurotransmitter receptors that share common structural features and activate common intracellular signaling systems. In a series of gene transfer experiments, they have demonstrated that although in neurons the 5HT receptors are involved in neurotransmission via regulation of ion channel function, in fibroblasts the same receptor alters the growth properties of cells and when overexpressed can lead to malignant transformation. Thus the distinct phenotypic consequences of receptor activation in fibroblasts and neurons may reflect the different ways in which different cell types are programmed to respond to the same set of signaling events.

The research of Investigator Thomas M. Jessell, Ph.D. (Columbia University) and his colleagues has focused on the cellular and molecular mechanisms that determine neuronal cell fate and the patterning of neuronal connections in the vertebrate spinal cord. Neuronal differentiation and axon guidance have been shown to be regulated by a group of specialized epithelial cells that occupy the ventral midline of the spinal cord. This cell group, which forms the so-called floor plate, releases a retinoic acid–like morphogen that polarizes embryonic tissues. At early stages of neural tube development, the floor plate may therefore play a role in the determination of cell identity and differentia-

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tion. At later stages the floor plate guides developing axons in the embryonic spinal cord, in part by releasing a diffusible chemoattractant factor and in part by virtue of its specialized cell surface properties. There are marked changes in the expression of glycoproteins on the surface of axons as they pass through the floor plate. To study the regulation of these axonal glycoproteins, cDNA clones encoding a recently discovered protein of this type, TAG-1, have been isolated. TAG-1 belongs to the immunoglobulin gene family and may be involved in cell recognition and axonal pathfinding in the developing central nervous system.

A major aim of the laboratory of Investigator Corey S. Goodman, Ph.D. (University of California at Berkeley) is to understand the molecular mechanisms that control how neuronal growth cones find and recognize their correct targets during development. Neuronal growth cones can navigate over long distances by following signals on the surfaces of cells (both glia and other neurons) and in the extracellular matrix. The major aim of this work is to uncover the adhesion, recognition, and signaling molecules that impart specificity to the developing nervous system and in so doing allow growth cones to recognize differentially their correct pathways and targets. Molecular genetic and classical genetic approaches are used to study the function of these molecules in the fruit fly, *Drosophila melanogaster*. Over the past year a number of *Drosophila* neural adhesion molecules have been cloned, and a detailed analysis of their function is under way.

Assistant Investigator Flora Katz, Ph.D. (University of Texas Southwestern Medical Center at Dallas) and her colleagues are interested in the mechanisms by which cell surface interactions in the nervous system govern differentiation and the establishment of neural networks. They have been studying a neural-specific cell surface carbohydrate modification found on many membrane proteins in *Drosophila*. Extreme disruptions of development occur at the nonpermissive temperature in a cold-sensitive mutant [*nac* (*neurally altered carbohydrate)*] in which this modification is absent. It is hoped that study of the biochemistry, molecular biology, and cellular and organismal phenotypes of this mutant will contribute to an understanding of the role of tissue-specific glycosylation in development.

Research in the laboratory of Investigator Gerald M. Rubin, Ph.D. (University of California at Berkeley) is directed toward examination of differentiation and gene regulation in the developing nervous system by studying certain genes whose mutation disrupts neural development. During the past year Dr. Rubin and his colleagues have focused on several genes important for the determination of cell fates in the developing retina of *Drosophila*. Among these are genes encoding cell surface receptors with structures similar to the mammalian insulin and epidermal growth factor receptors, and regulatory proteins that resemble mammalian DNA-binding proteins. Their observations suggest a striking evolutionary conservation of basic developmental mechanisms from flies to humans.

Associate Investigator Stephen J. Smith, Ph.D. (Yale University) and his colleagues are studying the development and function of synaptic connections. They are especially concerned with the basic motility mechanisms that enable growing nerve fibers to locate their proper targets for synapse formation. Other projects have led to the collection of video sequences of developmental events within intact mammalian brain tissue. These new observations concern mechanisms of neuronal migration and synapse formation during embryonic brain development. Still other projects address the detailed dynamics of intracellular signaling underlying the regulation of synaptic development and function.

Physical growth and retraction of nerve terminals cause changes in the circuitry of the brain. Development, repair, and memory depend on this plasticity. One neuronal protein implicated by several laboratories in this process is GAP-43. Associate Investigator Mark C. Fishman, M.D. (Massachusetts General Hospital) and his colleagues have investigated how GAP-43 is transported through the cell to discrete regions of the membrane. GAP-43 was found to contain a short stretch of amino acids that guides it to specific sites, including the nerve terminal. Once there it apparently enhances the extension of filopodia—long, fine processes from the cell surface—that normally characterize growing nerve terminals. GAP-43 appears also to interact with other proteins at the membrane, some of which are known to be important for the transduction of information from the cell surface into intracellular messages. These other proteins are being isolated to see how they mediate a linkage between cell surface signals and cell shape changes. Finally, a brain-specific protein has been identified and cloned that binds to the regulatory region of the GAP-43 gene.

Potassium channels are a diverse group of ion channels that are widely distributed in animal and plant cells and serve many different functions, including, possibly, learning and memory. The first

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potassium channel gene (*Shaker*) has been cloned in the laboratory of Investigator Lily Y. Jan, Ph.D. (University of California at San Francisco) from *Drosophila*. The strong conservation of potassium channel structure has allowed the subsequent isolation of mammalian potassium channel genes. Current studies reveal that one gene in *Drosophila* may give rise to multiple subtypes of potassium channels by alternative splicing. Structural components important for specific aspects of potassium channel function have also been identified by site-directed mutagenesis experiments.

Work in the laboratory of Investigator Paul R. Adams, Ph.D. (State University of New York at Stony Brook) focuses on the electrical properties of vertebrate nerve cells. The research addresses three main issues: What voltage-dependent channels are present? How are these channels regulated by membrane potential, intracellular calcium, and neurotransmitters? What roles do these channels play in cell physiology? This work is being pursued using several different types of nerve cells: bullfrog sympathetic ganglion cells, hippocampal pyramidal cells, lateral geniculate cells, and a clonal cell line.

The laboratory of Associate Investigator Robert G. Johnson, Jr., M.D., Ph.D. (University of Pennsylvania) is interested in the application of sensitive, rapid, and noninvasive biophysical techniques to study the cellular and molecular regulation of molecules within biological membranes that regulate the distribution of ions and chemical messengers across the plasma and intracellular membranes, specifically the Na^+ pump, nicotinic acetylcholine receptor, and a voltage-dependent calcium channel in secretory neuroendocrine cells. During the past year the laboratory has applied nuclear magnetic resonance spectroscopy to the study of the cellular regulation of the Na^+ pump and found that it is possible to measure simultaneously the ATPase activity and transmembrane Na^+ distribution. Using this technique and observing these parameters under resting and stimulated conditions, Dr. Johnson and his colleagues have shown that the activity of the Na^+ pump in an excitable cell can increase dramatically by a mechanism that is independent of the intracellular sodium concentration.

How do genes control animal development? Taking a primarily genetic approach to answer this question, Investigator H. Robert Horvitz, Ph.D. (Massachusetts Institute of Technology) and his colleagues have isolated developmental mutants of the nematode *Caenorhabditis elegans* and have used both genetic and molecular genetic techniques to characterize these mutants. Because both the complete cellular anatomy (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. In this way, genes involved in cell lineage, cell death, cell migration, and cell differentiation have been identified and analyzed.

Research in the laboratory of Assistant Investigator Gary Struhl, Ph.D. (Columbia University) is directed toward determining the molecular nature and mode of action of spatial information responsible for organizing the embryonic body plan of *Drosophila*. Genes encoding several molecules that behave as spatial cues have been identified and their functions assessed by a variety of approaches. These studies have recently led to the demonstration that different strategies are used to dictate how the anterior and posterior halves of the body develop: the anterior half depends on a single gradient morphogen, *bicoid*, which functions as a concentration-dependent transcriptional activator, while the posterior half depends on a number of local gradients that arise in response to the polarized activities of *bicoid* and two other primary morphogens, *nanos* and *torso*.

The nervous system consists of an enormous number of different cell types, and one of the central issues in neuroscience is how each neuron acquires its distinctive identity. In the past few years the laboratory of Investigator Yuh Nung Jan, Ph.D. (University of California at San Francisco) has identified in *Drosophila* more than 20 genes that bear the question of the specification of neuronal identity. During the past year the group has studied in detail the genes *neuralized, big brain, daughterless, cut, cyclin A, rhomboid,* and *numb*. All have now been cloned, most by this group. The studies are revealing clues as to how these genes affect neural development at the molecular level.

Investigator Louis F. Reichardt, Ph.D. (University of California at San Francisco) and his colleagues are investigating some of the molecules in the extracellular environment that regulate the survival and development of neurons *in vivo*. Among these are trophic factors that play a key role in neuronal survival. These are proteins that are made in minute amounts by cells; to date the best understood is nerve growth factor, which regulates the survival and differentiation of several neuronal populations, including a population of acetylcholine-secreting neurons in the brain that are important in cogni-

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tion. Dr. Reichardt is also studying certain molecules on the surfaces of cells and in the extracellular matrix that serve as substrates for guiding the growth of axons. A third project seeks to identify molecules that direct neurons to form synapses with other cells. Since synapses are the sites of information transfer between neurons, these molecules are also crucial in establishing the correct wiring of the nervous system.

Understanding the molecular mechanisms by which the neuroendocrine system develops and by which neuropeptides and hormones control critical regulatory events is the central goal of the laboratory of Investigator Michael G. Rosenfeld, M.D. (University of California at San Diego). Analysis of several hormone receptors has provided specific insights into the molecular mechanisms of signal transduction and hormonal regulation of gene expression and has led to the identification of a class of transcriptional activators that exhibit precise temporal and spatial patterns of expression in the developing nervous and endocrine systems. An unexpected mechanism for post-transcriptional regulation in the brain has been mechanistically defined by the analysis of a gene encoding the calcitonin gene-regulated peptide, which is the most potent known vasodilator.

Investigator Larry W. Swanson, Ph.D. (The Salk Institute for Biological Studies) and his colleagues have been characterizing the functional organization of brain circuits that determine behavioral state in mammals. Recent work has focused on the mechanisms that underlie some of the dramatic effects of the gonadal steroid hormone estrogen on the levels of a number of neuropeptides in neurons involved in reproductive behavior and physiology. They have also recently mapped the distribution of nicotinic acetylcholine receptors in the brain and, with Dr. Rosenfeld's group, have been localizing, within the brain, certain transcription factors that regulate gene expression.

The exquisite sensitivity and specificity of the olfactory system in the detection and discrimination of odors is remarkable. Associate Investigator Randall R. Reed, Ph.D. (The Johns Hopkins University) and his colleagues have identified several of the proteins believed to be responsible for signal transduction in the olfactory system. Olfaction and vision appear 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. Dr. Reed's laboratory has recently identified cDNA clones that encode several distinct forms of this enzyme. One of these, type III cyclase, is exclusively expressed in olfactory sensory neurons. To elucidate the role of the various forms of the mammalian enzyme, the group has identified homologues of several forms of adenylyl cyclase in the genetically manipulable organism *Drosophila*.

Sex pheromones are specific chemicals that are released by certain animals in order to trigger innate mating behavior. The ability of the target animal to respond to minuscule amounts of pheromone depends on a highly sensitive detection system. This sensory system is under investigation by Assistant Investigator Michael R. Lerner, M.D., Ph.D. (Yale University) and his colleagues. A second interest of the laboratory is in the control of gene expression, particularly in the central nervous system, by differential pre-mRNA splicing. Small nuclear ribonucleoproteins (snRNPs) are intimately associated with RNA processing in general. Many snRNPs in the central nervous system contain protein, which appears to replace the general snRNP B protein. How this protein contributes to the mechanism of alternative splicing is being explored.

The laboratory of Investigator King-Wai Yau, Ph.D. (The Johns Hopkins University) has continued its effort to understand the visual transduction process by which light triggers an electrical signal in retinal photoreceptors. Recently they have been engaged in two kinds of studies. First, in an attempt to clarify a long-standing puzzle in vision, they have made a comprehensive study of the adaptation behavior of rods in a variety of mammals and have found, contrary to widespread belief, that these cells behave much like those in lower vertebrates. Second, they have carried out an in-depth biophysical study of the gating characteristics of the ion channel that mediates phototransduction in rods and cones. This work has provided kinetic information about the ion channels that are directly activated by cyclic nucleotides, a unique and novel class of ion channels that includes the phototransducing channel.

The laboratory of Associate Investigator James B. Hurley, Ph.D. (University of Washington) continues to investigate the relationship between the biochemical properties of signal transducing G proteins and the physiological responses of the cells in which they are expressed. The group is comparing signal transduction pathways of retinal rods and cones to determine how differences between the signal transduction enzymes in these cells determine their light sensitivity and response kinetics.

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Dr. Hurley is also using molecular, immunological, and genetic methods to study G proteins in *Drosophila*. Several unique G proteins have been identified, and transformation experiments will be used to determine how their properties determine development and physiological responses of a whole organism.

The laboratory of Associate Investigator David P. Corey, Ph.D. (Massachusetts General Hospital) has made further progress in understanding the molecular mechanism of sensory perception by the receptor cells of the inner ear. The generally accepted hypothesis for the transduction mechanism is that there are ion channels in the tips of mechanically sensitive cilia that are directly activated by tension in fine filamentous links between the cilia. An adjustment or adaptation process is thought to involve some molecular motor that continually adjusts the tension in the filaments. They have found that the adjustment rate is constant when the filaments are thought to be slack, consistent with the hypothesis. They have also found that the voltage changes that are thought to alter the tension cause submicroscopic movements of the cilia with the appropriate time course. Biochemical experiments are under way to separate the protein constituents of the cilia, to identify those that might be involved in the transduction and motility processes.

Recent studies by Investigator Ronald M. Evans, Ph.D. (The Salk Institute for Biological Studies) and his colleagues have led to significant advances in our understanding of how steroid, retinoid, and thyroid hormones act to exert their regulatory effects on development and physiologic homeostasis. Although these hormones are structurally and biosynthetically distinct, cloning studies have shown that their receptors are members of a superfamily of regulatory genes that act as transcription factors. As such these receptors are of great interest for studying the mechanisms of transcriptional control. The various receptors display considerable specificity and selectivity in the genetic programs that they ultimately influence. This modulation of gene expression leads to profound changes in protein synthesis within cells and consequent changes in cell function. It is the combination of these final changes that ultimately determines the physiologic effect of the relevant hormones. The study of these molecules can be used to provide a rather direct link between molecular events in the genome and physiological events in the organism.

THE ROLE AND CONTROL OF IONIC CHANNELS IN EXCITABLE CELLS

PAUL R. ADAMS, PH.D., *Investigator*

Work in Dr. Adams's laboratory is focused on the electrical properties of vertebrate nerve cells. Research addresses three main issues. What voltage-dependent channels are present? How are these channels regulated by membrane potential, intracellular calcium, and neurotransmitters? What roles do these channels play in cell physiology? Several different types of nerve cells are being used: bullfrog sympathetic ganglion cells, hippocampal pyramidal cells, lateral geniculate cells, and a clonal cell line.

I. Bullfrog Sympathetic Ganglion.

M current is a voltage-dependent potassium current that influences firing adaptation in many vertebrate neurons and is synaptically regulated. In the past year, several issues relating to this current have been clarified. FURA imaging has been used to show that agents that increase intracellular calcium also inhibit M current, suggesting that calcium may play a second messenger role. However, experiments using photolysable calcium buffer show instead that small increases in intracellular calcium actually increase M current. M current increases in two other situations: after washout of muscarine and after intracellular perfusion with GDPβS. It is unclear if there is any connection between these three increases. Only when calcium is increased to high levels does M current decrease. This effect resembles that produced by phorbol esters, in that the residual M current becomes muscarine insensitive.

Although these experiments complicate possible hypotheses about second messengers, other work has strengthened the idea that second messengers are involved. Cell patch recordings have revealed a voltage-dependent potassium channel that gives M-like ensemble averages. This putative M channel is active in the expected range of membrane potentials and in a significant fraction of trials is turned off by muscarine applied to the rest of the cells but not by muscarine in the patch electrode.

The involvement of intracellular calcium in other membrane responses has also been pursued. Confocal microscopy, FLUO imaging, and whole-cell recording have been combined to examine the dynamics of calcium after a brief activation of plasmalemmal calcium channels. Calcium equilibrium throughout the cytoplasm takes ~300 ms. This is followed by a slowly subsiding, spatially uniform signal. A detailed computer simulation shows that this process is accomplished mainly by diffusion of calcium-loaded buffers. Considerable progress has also been made in understanding the kinetics of calcium removal by buffering, uptake into intracellular stores, mitochondrial uptake, and extrusion.

II. Hippocampal Slices.

The laboratory has previously identified or clarified the role of several ionic currents in these cells (I_M, I_Q, I_{AHP}, and I_C). More recent work has turned to transient outward currents previously lumped together as I_A. It now appears that there are two separate 4-aminopyridine–sensitive transient currents operating in a similar voltage range. The fastest is a true A current, while the lower one, I_D, is responsible for the delayed firing that is seen after weak stimuli at hyperpolarized membrane potentials.

III. Lateral Geniculate Nucleus (LGN).

Initial work in rodent LGN characterized transient voltage-dependent currents underlying cell firing in relay cells. Work has now shifted to feline LGN, where different classes of relay cell can be readily distinguished. Experiments are being performed to analyze the contributions of NMDA channels and T channels to optic tract evoked synaptic potentials.

IV. PC12 Cells.

Efforts to identify biochemical pathways controlling ion channels in sympathetic neurons have been hampered by unsuitability of the tissue. To overcome this problem work, Dr. Adams and his colleagues have initiated work with a chromaffin cell line that differentiates to form neuron-like cells. These cells possess M current, which is coupled to activation of bradykinin receptors.

Dr. Adams is also Professor of Neurobiology and Behavior at the State University of New York at Stony Brook.

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Books and Chapters of Books

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EXPRESSION OF NEURAL GENES

SUSAN G. AMARA, PH.D., *Assistant Investigator*

Dr. Amara is concerned with the molecular biology and regulation of genes encoding proteins with important roles in neurotransmission. Neurotransmitter transporters have a central role in synaptic transmission and are the site of action for a wide range of clinically important drugs. Most recently the laboratory has pursued a molecular characterization of the proteins responsible for neurotransmitter reuptake and has developed a functional expression system for identifying the genes encoding these carriers. Other investigations have continued to explore the basic mechanisms for regulating both neuronal gene transcription and alternative pathways of pre-mRNA processing, using a neuropeptide gene family as a model system.

I. Neurotransmitter Transporters.

Despite a plethora of pharmacologically impor tant inhibitors of monoamine uptake (including cocaine, amphetamines, and the tricyclic antidepressants), little molecular detail is known about the proteins responsible for reuptake of neurotransmitters at these synapses. Even for acidic amino acid transporters, which are relatively more abundant in brain and have been the focus of detailed bioenergetic and kinetic studies, little structural information is available, despite the significant roles these activities play in synaptic function and signal termination in the nervous system. Dr. Amara and her colleagues have been using microinjection of mRNA into *Xenopus laevis* oocytes to express four major classes of brain transport activity: catecholamine, indoleamine, choline, and excitatory and inhibitory amino acid transport. After injection of mRNA prepared from various brain regions, uptake of the radiolabeled transmitters can be measured in single oocytes and displays a pattern of regional distribution consistent with the known anatomical location of neurotransmitter-synthesizing cell bodies. Sodium dependence and pharmacologic specificity of uptake can also be assessed in oocytes; these studies have confirmed that the transport activities induced by brain mRNA display properties very similar to the high-affinity, sodium-dependent transporters observed in brain slices and synaptosomal preparations.

Studies on the L-glutamate carrier expressed from cerebellar mRNA have permitted the characterization of a transport activity from a population of well-defined glutamatergic neurons, the cerebellar granule cells. In these studies L-glutamate transport was observed to be sodium- and time-dependent, temperature sensitive, and saturable at micromolar substrate concentrations. In addition, cerebellar mRNA-induced glutamate uptake was inhibited by compounds known to block high-affinity uptake in brain slices and synaptosomes. Cerebellar forebrain and brain stem RNAs have been size-fractionated on sucrose gradients and assayed for transport of L-glutamate and GABA (γ-aminobutyric acid). These studies reveal single, comigrating, pharmacologically distinguishable peaks for L-glutamate and GABA transport, with maximal activity observed in fractions containing RNA in the 2.5–3.0 kb range. In contrast, both L-glutamate and GABA transport activities in forebrain appear to be encoded by larger mRNA species. Dihydrokainate fails to inhibit the cerebellar mRNA-induced L-glutamate transport, while significantly reducing forebrain and spinal cord mRNA-induced transport, supporting the presence of at least two forms of sodium-dependent L-glutamate transporters. Developmental studies have demonstrated substrate- and region-specific postnatal increases in L-glutamate, GABA, and glycine transport activity. The identification of the genes encoding these transporters will provide a basis for future studies on their heterogeneity and distribution and on the molecular mechanisms of transporter function and specificity; such experiments are in progress.

II. Neuropeptide Gene Regulation.

Previous studies on the structure and expression of the calcitonin/α-CGRP (calcitonin gene-related peptide) gene demonstrated several of its unique features. Alternative pre-mRNA splicing within coding domains generates two mRNAs: calcitonin mRNA is made within the thyroid and α-CGRP mRNA is made in a variety of neuronal cell types. The gene consists of six exons: three upstream exons used in both mRNAs, a fourth exon containing the calcitonin mRNA-specific domain and polyadenylation site, and two downstream CGRP mRNA-specific exons followed by a second polyadenylation site. Another form of CGRP, β-CGRP, is encoded on a distinct gene. The calcitonin/α-CGRP and β-CGRP genes code for two nearly identical

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neuropeptides that are expressed differentially and in discrete regions in the nervous system.

These independently regulated genes offer an excellent model system in which to explore the determinants of tissue-specific gene expression. Dr. Amara and her colleagues have examined the regulatory role of 5′ upstream sequences of α- and β-CGRP genes by analyzing the expression of gene constructs introduced into cells after transient DNA transfection. Promoter regions have been fused to reporter genes. Constructs containing α- and β-CGRP 5′-flanking regions direct the expression of chloramphenicol acetyltransferase (CAT) activity in several cell lines derived from neural, endocrine, and nonneural tissues. Constitutive α- and β-CGRP promoter-driven expression is low relative to expression from a strong viral promoter (SV40) but can be induced severalfold after exposure of cells to various hormonal agents. Deletion mapping of the upstream promoters is being utilized to delineate further the functional regions that mediate observed hormonal effects.

III. Regulated Pre-mRNA Processing.

Alternative pre-mRNA processing is an important mechanism for regulating the diversity of gene products. A wide array of gene products are encoded within complex transcription units that generate multiple mature mRNAs through the selective use of multiple start sites, splice sites, and/or polyadenylation sites. Whether the crucial regulatory events of the calcitonin/α-CGRP gene involve the selective use of polyadenylation sites, the selective use of 3′ splice sites, or both, is controversial. It is equally unclear whether a specific trans-acting factor regulates the CGRP splice choice, with calcitonin produced by a default pathway, or vice versa, or whether each pathway requires specific factors. Having established the baseline pattern of mRNAs produced in several cell lines, Dr. Amara and her colleagues have also introduced several mutated and hybrid genes into cultured cells to identify sequences critical for splice regulation.

The mechanism of pre-mRNA processing regulation can be considered from two perspectives, each of which suggests an experimental approach. One determinant of a particular processing choice clearly resides within the primary RNA transcript, either as a specific sequence or secondary structural feature. The other determinant resides in the enzymatic machinery that recognizes these specific structural features and targets the transcript to a particular processing pathway. For regulated processing events, tissue-specific differences in the basic processing machinery must be postulated.

Although transfection and mutagenesis studies should provide insight into the cis-acting sequences that influence alternative processing, a major goal is to learn more about the trans-acting factors that influence these events. Therefore, studies have been directed toward establishing a convenient assay system in which to test effects of exogenously added factors on the splicing patterns. One approach has been to microinject the calcitonin/α-CGRP gene into the *Xenopus laevis* oocyte nucleus and analyze the RNA products that result. The gene constructs used for these experiments contain the calcitonin/α-CGRP gene under the control of its own promoter or, alternatively, fused to a viral promoter known to work well in the oocyte. The pattern of basal expression of the calcitonin/α-CGRP gene suggests that the oocyte nucleus transcribes the gene well but lacks the machinery necessary to make the regulated processing steps.

Studies on tissue-specific snRNPs, snRNAs, and putative factors involving alternative pre-mRNA processing continue as a collaborative project with Dr. Michael R. Lerner (HHMI, Yale University School of Medicine). Co-injection experiments using exogenous nuclei, nuclear extracts, enriched snRNP preparations, or snRNAs are under way to identify specific factors that influence processing events.

Dr. Amara is also Assistant Professor of Molecular Neurobiology at Yale University School of Medicine.

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STRUCTURE AND FUNCTION OF THE SEROTONIN RECEPTORS

RICHARD AXEL, M.D., *Investigator*

Serotonin is a biogenic amine that functions as a neurotransmitter, a hormone, and a mitogen. Serotonin-containing neurons project to most regions of the mammalian central nervous system and mediate diverse neural functions. In the brain, serotonergic synapses regulate sensory motor reflexes and modify cortical circuits involved in affective functions. In the periphery, serotonin activates chemoreceptive sensory endings and controls enteric reflexes. This diverse set of physiologic processes results partly from the interaction of serotonin with distinct receptors. Multiple serotonin receptors have been defined on the basis of ligand-binding properties. Moreover, individual receptor subtypes activate different intracellular signaling systems. The 5HT1c and 5HT2 receptors activate phospholipase C, whereas 5HT1a and 5HT1b receptors regulate adenylate cyclase or couple to G proteins that activate ion channels directly.

I. Structure of Serotonin Receptors.

In collaboration with Dr. Thomas M. Jessell (HHMI, Columbia University College of Physicians and Surgeons), Dr. Axel's laboratory has cloned and characterized the structure and function of two serotonin receptors, in an attempt to elucidate the mechanism of action of serotonin in the mammalian nervous system. A cDNA expression system was designed that permits the identification of functional cDNA clones encoding transmitter receptors in the absence of protein sequence information. By combining cloning and RNA expression vectors with an electrophysiologic assay in oocytes, they have isolated functional clones encoding both the 5HT1c and 5HT2 receptors. The cDNAs of both receptors encode proteins that share numerous sequence and structural properties with the family of receptor molecules predicted to span the lipid bilayer seven times. The 5HT2 receptor shares an overall sequence identity of 49% with the 5HT1c receptor, but the amino acid identity within the transmembranes is 80%. The extent of sequence identity may reflect either a very recent gene duplication or evolutionary conservation required to maintain a common function. Comparison of the 5HT1c and 5HT2 sequences, however, shows extensive divergence in the amino- and carboxyl-terminal regions, as well as in the third cytoplasmic loop. These observations suggest that the two genes do not result from a recent duplication event but that the stringent conservation is required to maintain a set of functions shared by the two receptor subtypes. The striking conservation of sequence within the transmembrane domains is consistent with the observation that the two receptors exhibit common ligand-binding properties and couple to the same intracellular signaling pathway. Thus these two serotonin receptors define a new family of G protein–coupled receptors.

II. Function of Serotonin Receptors in Novel Cellular Environments.

Previous studies have suggested that the 5HT1c and 5HT2 receptors activate phospholipase C in cells of the central nervous system. The genes encoding these receptors were therefore introduced into novel cellular environments to obtain additional evidence that the two receptors activate common intracellular signaling systems. In *Xenopus* oocytes injected with either 5HT2 or 5HT1c receptor RNA, serotonin elicits a rapid inward current that is reversibly blocked by selective 5HT antagonists. This inward current is likely to be triggered by receptor-mediated activation of phospholipase C and the subsequent release of inositol phosphates. These second messengers promote the mobilization of intracellular calcium and the opening of calcium-dependent chloride channels. These experiments demonstrate that the 5HT2 and 5HT1c cDNAs encode functional serotonin receptors that confer serotonin sensitivity to *Xenopus* oocytes.

A separate series of experiments demonstrated that the 5HT1c and 5HT2 receptors are functional when introduced into NIH 3T3 fibroblasts. Fibroblasts expressing either receptor on their cell surface exhibit specific high-affinity binding with serotonin. The functional response of transfected cells to serotonin was analyzed by loading cells with the calcium-sensitive dye Indo I and monitoring changes in the fluorescent emission spectrum of the dye in a flow cytometer. Cells transformed with either the 5HT1c or 5HT2 receptor cDNA reveal a marked increase in intracellular calcium when exposed to serotonin. These experiments indicate that this family of 5HT receptors couples to the same intracellular signaling systems in neurons, fibroblasts, and *Xenopus* oocytes.

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III. 5HT1c and 5HT2 Receptors Function as Proto-oncogenes in Fibroblasts.

Although fibroblasts express intracellular signaling pathways activated by the 5HT1c and 5HT2 receptors, this second messenger pathway is likely to couple to effector proteins that differ from those in neurons and that may mediate distinct cellular functions. Dr. Axel's laboratory therefore examined the functional consequences of activating these receptors in NIH 3T3 fibroblasts. These studies demonstrated that 3T3 cells expressing high levels of either the 5HT1c or 5HT2 receptor form foci in cell culture. Several observations indicate that the formation of foci results from the expression and activation of 5HT receptors on the cell surface. First, the introduction of functional 5HT1c or 5HT2 receptor cDNA into NIH 3T3 cells results in the generation of transformed foci at high frequency. Foci are not observed after transfection with expression vectors lacking the 5HT receptor cDNA. Second, cells within transformed foci exhibit a high density of functional 5HT receptors on the cell surface. Third, the generation of foci after DNA transfer is completely blocked by serotonin antagonists. Fourth, transformed foci expressing the 5HT receptors fail to reestablish foci when plated in serotonin antagonists, such as mesulergine. Finally, the injection of cells derived from transformed foci obtained after expression of the 5HT1c receptor into nude mice results in the generation of tumors. Thus activation of the same receptor in fibroblasts and neurons elicits distinct phenotypes. In neurons the 5HT receptors are involved in neurotransmission via regulation of ion channel function, whereas in fibroblasts the same receptor alters the growth properties of cells and results in malignant transformation. The distinct phenotypic consequences of receptor activation in fibroblasts and neurons may reflect the different ways in which different cell types are programmed to respond to the same set of signaling events.

The observation that expression of 5HT1c and 5HT2 receptors elicits focus formation only in the presence of ligand permits the selection of mutants affecting components of the signal transduction machinery. This receptor-mediated transformation system should facilitate a somatic cell genetic analysis of neurotransmitter-mediated signaling.

IV. Activation of the β-Adrenergic Receptor Promotes Growth and Differentiation in Epithelial Cells.

Studies with serotonin receptors indicate that a single ligand may interact with the same receptor subtype in different cells to elicit distinct cellular responses. The β_2-adrenergic receptor was therefore introduced into the unnatural environment of the thyroid cell to demonstrate that the activation of this receptor also initiates diverse cellular programs in different cell types. The cell line FRTL5 is a continuous line of thyroid cells that depends on thyroid-stimulating hormone (TSH) for growth and differentiation. Activation of the TSH receptor stimulates adenylate cyclase, resulting in an increase in cAMP. The β_2-adrenergic receptor also stimulates adenylate cyclase, but this receptor subtype is not expressed in thyroid cells. Experiments were performed to ask whether thyroid cells transfected with cDNA encoding the β_2-adrenergic receptor will undergo growth and differentiation in response to the adrenergic ligand isoproterenol. In thyroid cells transfected with the β_2-adrenergic receptor, isoproterenol elicits the same program of thyroid-specific functions observed with TSH. The β_2-adrenergic receptor that contributes to autonomic neurotransmission in the sympathetic nervous system regulates growth and differentiation in the unnatural environment of a thyroid cell. Thus the functional distinction between a neurotransmitter receptor and a growth factor (or even an oncogene product) may depend critically on the cellular environment.

Dr. Axel is also Higgins Professor of Biochemistry and Pathology at the Columbia University College of Physicians and Surgeons.

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ACTIVATION AND REGULATION OF ION CHANNELS IN NEURAL CELLS

DAVID P. COREY, PH.D., *Associate Investigator*

Dr. Corey's laboratory is interested in the regulation of membrane permeability in neural cells, which underlies such processes as sensory transduction, membrane excitability, and synaptic transmission. The goal is to understand the ion channel proteins that mediate permeability, the mechanism of their activation, and the processes of their expression and regulation. Ion channels are being studied in two different systems: in hair cells (the mechanically sensitive receptor cells of the inner ear) and in glial cells of the optic nerve.

I. Mechanism of Sensory Transduction in Hair Cells.

The ion channels that mediate auditory and vestibular sensory reception are thought to be activated directly by mechanical stress applied to them via fine filaments. Increased tension in the filaments, which stretch between adjacent cilia, corresponds to increased probability of opening. Progress has been made toward understanding this mechanism and its regulation by an adaptation process.

A. *Rate and calcium control of the adaptation mechanism.* During a maintained positive displacement there is a decline or adaptation in transduction current; this seems to result from a relaxation of the gating spring. Similarly, a displacement that allows channels to close activates a retensioning mechanism that reopens channels; both are such as to keep constant the steady-state, channel-open probability. Dr. Corey and his colleagues had previously found that the relaxation process is much faster than tensioning and that the tensioning rate and static tension are similar to those of myosin moving on actin in other systems. These and other data suggested a calcium-regulated, myosin-like motile system that regulates string tension.

With a new preparation of dissociated, patch-clamped hair cells, Dr. Corey and his colleagues have found that the rate of tensioning becomes independent of the position of the bundle, if the bundle is moved far enough that the filaments are expected to be slack. This suggests that the motile element underlying adaptation is attached to the filaments.

The relation between displacement and channel opening [the P(X) curve] was also found to be voltage-dependent; this was believed to follow indirectly from the voltage dependence of calcium entry and the calcium dependence of the adaptation rates. If the filament hypothesis of transduction is correct, then an unrestrained bundle should move when the membrane potential is changed, since potential apparently modulates the tension in the filaments. This was observed: average movements were ~40 nm, very close to the value predicted from the P(X) shift and the bundle stiffness. In addition, the time course of the motion has been studied, and its asymmetry and time constants agree with the idea that the motion is produced by the same motile element.

B. *Protein constituents of purified stereocilia.* To understand transduction or adaptation at a molecular level, it is necessary to know what proteins are involved in these mechanisms. Dr. Corey's laboratory developed a method, using nitrocellulose adhesion, for the rapid and efficient isolation of hair cell stereocilia from bullfrog sacculus. Polyacrylamide gels revealed about a dozen protein bands; actin, fimbrin, calmodulin, and calbindin are among them. The further localization and identification of these proteins has been studied by producing detergent-insoluble cores of stereocilia; these contain actin, fimbrin, and three unidentified proteins, with molecular weights between ~45 and 60 kDa.

II. Function of White Matter Glia.

The function of optic nerve glial cells and their interactions with retinal ganglion cells are being investigated by Dr. Barbara A. Barres in Dr. Corey's laboratory. The optic nerve preparation is used because it has a comparatively simple structure. In addition to ganglion cell axons, three glial cell types are present: type 1 astrocytes, type 2 astrocytes, and oligodendrocytes.

A. *Ion channel phenotype of glial cells* in vivo. Using whole-cell and single-channel patch recording, Dr. Barres has found that each optic nerve glial cell type expresses a unique set of ion channel types in culture. These phenotypes have begun to suggest certain hypotheses for glial function. A general concern in these studies is that the phenotype of cells in culture may not accurately represent that *in vivo*. For instance, type 2 astrocytes cultured in serum-containing medium express a charybdotoxin-sensitive potassium channel that is not expressed by type 2 astrocytes in serum-free medium. Which is the *in vivo* phenotype?

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To answer this, Dr. Barres has developed a "tissue print" preparation that allows acute isolation of optic nerve glia for electrophysiological studies. Enzymatically treated tissue is gently pressed against a nitrocellulose-coated surface and removed, leaving a thin layer of adherent, viable cells still bearing processes. Retention of processes is important, because some ion channel types appear to be localized to processes. For instance, sodium channels are found in only ~20% of dissociated type 1 astrocytes (lacking processes) but in 100% of those in tissue prints. Each optic nerve glial cell type has now been studied in these tissue prints, and in some cases different ion channels are found in these cells than were found *in vitro*.

The next step in these studies of glial function is to reconstitute the *in vivo* phenotypes *in vitro*. In addition to the culture medium, the presence of certain cells may be essential for reconstitution. Sodium current is expressed by 100% of type 1 astrocytes in tissue prints but by only 15% of those in culture. However, if type 1 astrocytes are cocultured with purified retinal ganglion cells, then these astrocytes form processes and, in preliminary experiments, all develop sodium currents.

B. *The O2A glial progenitor cell.* The type 2 astrocyte and oligodendrocyte are generated by a common bipotential cell, the O2A progenitor cell. The electrophysiological properties of the O2A have been studied in a new serum-free culture system developed by Drs. Laura Lillien and Martin Raff. Dr. Barres has found that O2A progenitors in these cultures have many properties characteristic of neurons: they express the neuronal form of the sodium channel, they fire single regenerative potentials, they have glutamate-activated ion channels, and they synthesize the neurotransmitter γ-aminobutyric acid (GABA). Nearly identical properties were observed in acutely isolated O2A progenitors, indicating that this phenotype is not an artifact of culture. The O2A did not express a simple subset of channel types found in its descendant cells, the type 2 astrocyte and the oligodendrocyte, studied in the same culture system. Thus during development these active membrane properties may contribute to O2A function *in vivo*.

C. *GABA in glia.* Although GABA, a major inhibitory neurotransmitter in the brain, is thought to be synthesized solely by neurons, Dr. Barres has recently observed that O2A progenitors and type 2 astrocytes in culture express a surface antigen found on a subset of GABAergic neurons, recognized by the monoclonal antibody VC1.1. Antibodies to GABA also labeled these cells. The presence of GABA in these cultures was confirmed using high-performance liquid chromatography (HPLC). Because these cultures are both neuron- and serum-free and the culture medium does not contain GABA, these cells appear to be synthesizing it. However, Dr. Barres was unable to detect the presence of the GABA synthetic enzyme, glutamic acid decarboxylase, which in neurons converts glutamate to GABA. Instead, they appear to synthesize it by an alternative pathway that uses putrescine as a precursor. When putrescine was omitted from the culture medium, GABA-like immunoreactivity disappeared from both O2A progenitors and type 2 astrocytes, and GABA could no longer be detected by HPLC. Preliminary experiments suggest that this GABA synthesis is not an artifact of putrescine-containing tissue culture conditions: acutely isolated O2A lineage cells in tissue prints are labeled by GABA antibodies.

D. *Function.* The unique and complex electrophysiological properties of each optic nerve glial cell type and the presence of glutamate-activated ion channels and the neurotransmitter GABA in O2A progenitors and type 2 astrocytes suggest that glial cells—despite their passive reputations—may be capable of some surprisingly interactive behavior. Since type 2 astrocytes have processes that closely appose nodes of Ranvier, it appears plausible that some glial types may even participate in local modulatory circuits.

Dr. Corey is also Assistant Physiologist in the Department of Neurology, Massachusetts General Hospital, and Assistant Professor of Neuroscience at Harvard Medical School.

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MOLECULAR GENETICS OF STEROID AND THYROID HORMONE RECEPTORS

RONALD M. EVANS, PH.D., *Investigator*

A central question in eukaryotic molecular biology is how specific DNA-binding proteins bind regulatory sequences in target genes to influence cell function and fate. Dr. Evans and his colleagues have demonstrated that steroid, thyroid hormone, and retinoid (vitamin A) receptors are composed of a common structure and comprise a supergene family of ligand-dependent transcription factors. These receptors, in combination with their hormones, constitute critical regulators of complex developmental and physiologic processes. With regard to the products of this supergene family, one challenge is to understand the molecular properties of each receptor that determine its interactions with the transcriptional machinery regulating gene expression. A second, and perhaps more daunting, challenge is to understand the contribution of individual regulatory systems to the integrated and complex biologic responses inherent in development and homeostasis. These hormone receptor genes have been cloned and molecular techniques have been employed to characterize the structure-function relationships of individual receptor molecules, as the essential first step toward understanding higher levels of control.

I. Determinants of Target Gene Specificity.

The molecular specificity of the receptors for steroid and thyroid hormones is achieved by their selective interaction with DNA-binding sites, referred to as hormone response elements (HREs). HREs can differ in primary nucleotide sequence as well as in the spacing of their dyadic half-sites. Previously it was shown that the modular structure of the glucocorticoid and thyroid hormone receptors can be exploited in the study of DNA-binding specificity. *In vitro* mutagenesis has been used to interconvert the binding specificities of the steroid and thyroid hormone receptors. The target gene specificity of the glucocorticoid receptor can be converted to that of the estrogen receptor by changing three amino acids clustered in the first zinc finger region of the DNA-binding domain. A single Gly-to-Glu transition in this region produces a receptor that recognizes both glucocorticoid and estrogen response elements. Further replacement of five amino acids in the stem of the second zinc finger region transforms the specificity to that of the thyroid hormone receptor. These findings localized the structural determinants required for discrimination of HRE sequence and half-site spacing and suggest a simple pathway for the coevolution of receptor DNA-binding domains and hormone-responsive gene networks.

II. Retinoic Acid Receptor (RAR).

Retinoic acid, a metabolite of vitamin A, is capable of inducing a complex array of developmental and physiologic responses in vertebrate cells and tissues. The actions of retinoids are believed to be mediated through nuclear receptors, which modulate the expression of specific gene and target cells. Thus the discovery by Dr. Evans and his colleagues of the RAR offers, for the first time in a vertebrate system, the hope of analyzing the mechanisms of morphogenesis by identifying a set of developmentally controlled genes. Retinoic acid is known to have effects on the vertebrate limb pattern in development and regeneration, supporting a model in which a gradient of retinoic acid serves as a morphogen to supply positional information differentially to a developing limb. One prediction of this model is that the putative RAR must be expressed in the developing and regenerating limb anlage. The expression of the RAR has been investigated in the adult newt, whose amputated limbs are capable of regenerating and upon which retinoic acid can act to alter pattern. *In situ* hybridization studies confirm the presence of the RAR in the regenerating limb and localize expression specifically in the regenerating mesodermal cells that control limb pattern. It was also demonstrated that the RAR gene is not active in the adult limb, becomes activated after amputation (but only at the site of the stump) and remains active during the regeneration process, and then turns off once the limb is complete. These results indicate that the morphogenic field is established through differential activation of preexisting RARs. Despite the large amount of knowledge about the action of retinoids, specific genes that are activated by the receptor have yet to be identified. However, because of structural homology of the RAR to the thyroid hormone receptor, it was possible to demonstrate that both retinoic acid and thyroid hormones can induce gene expression through a common HRE. This induction is unexpected, because thyroid hormones and retinoids are proposed to elicit different effects

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on development and differentiation. However, these results suggest they may control overlapping in gene networks and lead to a reexamination of the relationships of these two hormones.

Two homologues of the human RAR in the fruit fly *Drosophila* have been identified. One locus, *knirps-related*, may carry out important events in establishing pattern formation in the early *Drosophila* embryo. *In situ* hybridization reveals that this gene is expressed in maternally derived RNA and is widely distributed, but in the early zygote transcript accumulation becomes localized. It is expressed in a broad anteroventral domain before the cellular blastoderm stage and then acquires two additional circumferential bands of expression after blastoderm formation.

A second *Drosophila* gene product, 2C, has been identified and is embedded in the genetically well-defined locus. This gene shares homology with a novel human receptor expressed during early embryonic development in vertebrates. This observation suggests that a new regulatory system might be identified in *Drosophila*, which could lead to the identification of a new human morphogenetic system.

III. Conditional Ablation in Transgenic Mice.

Transgenic animals have been created as a strategy to study vertebrate development. Tissue-specific promoters and enhancers make it possible to direct expression in cloned genes to restricted cell types, enabling the experimental manipulation of cellular and organ physiology. The reciprocal to this augmentation approach would be the creation of a transgenic hypomorph (i.e., a mouse deficient in a particular cellular function). Transgenic hypomorphs were created that result in the conditional ablation of specific cell types during defined periods of development and differentiation. Toxicity is based on the targeted expression of the herpes simplex virus thymidine kinase (HSV-TK) gene product. The ablation is induced by treating transgenic animals expressing this gene with the antiherpetic ganciclovir. In tissues of mice expressing HSV-TK, administration of ganciclovir leads to the rapid accumulation of toxic intermediates, disrupting cellular DNA replication and ultimately leading to rapid cell death. The ability to control and direct ablation allows for creation of conditional mutant phenotypes at precise periods of development. This technique also provides a potential means to enrich stem cell populations as well as permitting the creation of animal models for particular pathological conditions.

The thymidine kinase obliteration (TKO) approach has been employed to generate animals with controlled amino deficiency. After seven days of treatment with ganciclovir, animals expressing HSV-TK off an immunoglobulin promoter have greatly depleted B cells and are completely deficient in their T cell population.

In a similar study the HSV-TK gene has been expressed under control of the rat growth hormone or rat prolactin promoter. If transgenic mice expressing TK in somatotrophs are treated with ganciclovir, they develop as dwarfs. The anterior pituitary in these animals is nearly devoid of both somatotrophs and lactotrophs. By employing various concentrations of drug exposure and by initiating treatment at a variety of times after birth, Dr. Evans and his colleagues were able to conclude that both somatotrophs and lactotrophs derive from a common stem cell that exists in the adult and is capable of repopulating the pituitaries of treated animals with mature growth hormone and prolactin-producing cells.

Dr. Evans is also Professor, Gene Expression Laboratory, The Salk Institute for Biological Studies and Adjunct Professor of Biology at the University of California at San Diego.

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MOLECULAR BIOLOGY OF NERVE TERMINAL PLASTICITY

MARK C. FISHMAN, M.D., *Associate Investigator*

Regulated growth and retraction of nerve terminals mediate changes in neuronal connectivity during development, repair, and memory. Dr. Fishman and his colleagues are investigating the molecular basis of this plasticity. Over the recent years they have focused on the gene for the GAP-43 protein, since this gene is regulated concordantly with growth and the protein is enriched in growth cone membranes.

I. GAP-43 and the Growth Cone.

The previously cloned GAP-43 cDNA has been used to isolate and analyze the rat gene for GAP-43. Ed Grabczyk and Dr. Mauricio Zuber have determined some elements of the GAP-43 promoter: it lacks TATA or CAAT boxes, has multiple transcriptional start sites, and includes a region of H, or a triple-stranded, DNA. Adjacent to this region is a motif identical to that recognized by transcriptional regulators of the POU family. Dr. Sally Teng and Dr. Zuber have cloned a brain-specific protein that binds to this region. They are evaluating the role of the promoter's structure and this trans-acting protein in GAP-43 gene regulation. Other regions that are subject to regulation by, for example, corticosteroids or nerve growth factor (NGF), reside within introns or the 3′-untranslated region of the gene.

The function of GAP-43 is not known, although it has been speculated that it plays a role in nerve terminal plasticity. To establish a bioassay for GAP-43, in isolation from endogenous GAP-43 and other neuronal proteins, Dr. Zuber generated nonneuronal cell lines that express GAP-43 at high levels. In this context the expression of GAP-43 causes marked filopodial extension from the cell surface. Because these cells lack specific neuronal machinery, it is likely that GAP-43 can interact with structural components that are more universally expressed and that normally cause cell shape changes for mitosis or migration. This work suggests that the role of GAP-43 may be to regulate filopodial extension and permits mutational analysis of the regions of GAP-43 that are important to this phenotype. This assay is also being used to identify other molecular components that interact with GAP-43 to cause membrane remodeling.

In an attempt to understand these other parts of the growth cone transduction system, Dr. Stephen Strittmatter has isolated the major protein components of growth cone membranes. Two of the most prominent are the α- and β-subunits of G_o. Their specific subtypes were identified by microsequencing, in collaboration with Dr. Tim Kennedy. The G protein family functions to link membrane receptors to intracellular signaling systems, but the role of G_o, in particular, is poorly defined. Its growth cone enrichment suggests a role in that structure. Dr. Fishman's laboratory has also shown that GAP-43 and G_o are physically associated. The notion that a complex of proteins (including GAP-43 and G_o) constitute a chemical/mechanical transduction system that converts extracellular stimuli into directed growth is being tested.

GAP-43, although tightly associated with the growth cone membrane, bears no transmembrane domain in its structure. This suggests that it contains a domain responsible for enrichment in one particular region of the plasma membrane, the growth cone. This would be akin to "sorting sequences," short stretches of amino acids that determine whether proteins in epithelial cells accumulate on the basal or apical membranes. Drs. Zuber and Strittmatter have studied this possibility by mutational analysis and generation of chimeric proteins between pieces of GAP-43 and the cytosolic protein chloramphenicol acetyltransferase (CAT). The amino terminus of GAP-43 has been identified as responsible for membrane binding. This region causes chimeric proteins to accumulate in a distribution indistinguishable from that of GAP-43, which in neuronal cell lines includes the growth cone. The two amino-terminal cysteines are especially critical to this guidance. The possibility that accumulation occurs because the amino terminus binds to other proteins, perhaps GAP-43 "receptors" on the inner face of the plasma membrane, is being investigated. These studies suggest that GAP-43 bears a cellular trafficking domain that guides it to specific regions of the cell, especially those where remodeling occurs, where it interacts with membrane transduction systems to enhance filopodial extension.

II. Transgenic Models of Sensory Neuropathy.

Although it would be of great interest to evaluate the effects of disturbing gene expression in neurons, especially in their normal environment, it is

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not simple to introduce genes into neurons, because these cells are postmitotic. Dr. Fishman's laboratory has shown, in collaboration with Dr. Philip Leder (HHMI, Harvard Medical School), that the promoter of the calcitonin gene-related peptide (CGRP) gene functions in transgenic mice to direct expression of transgenes to subpopulations of sensory neurons and to the thyroid C cells. A variety of oncogenes have been found to be without effect when introduced in this manner. Dr. Manfred Baetscher has found that the SV40 large T antigen, however, not only causes tumors of the C cells, but also perturbs neuronal function, most evident phenotypically as an ataxic gait. Preliminary pathological analysis suggests that lysosomal debris accumulates within DRG neurons. Dr. Fishman and his colleagues are studying this inherited neuropathy to investigate how a dominant oncogene, which regulates cell cycle in other cells, perturbs function in postmitotic neurons.

Dr. Fishman is also Associate Professor of Medicine at Harvard Medical School and Assistant Physician at Massachusetts General Hospital.

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MOLECULAR GENETICS OF CELL ADHESION AND CELL RECOGNITION DURING NEURONAL DEVELOPMENT 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 are a focus of Dr. Goodman's laboratory. Previous studies have shown that neuronal growth cones can navigate over long distances and often through a series of complex choice points to find their correct targets; they appear to do so by following signals on the surfaces of cells (both glia and other axons) and in the extracellular matrix; evidence from other laboratories suggests that they can also follow diffusible gradients. One major aim is to uncover the adhesion, recognition, and signaling molecules that impart specificity on the developing nervous system and, in so doing, allow growth cones to recognize their correct pathways and targets differentially. Molecular genetic approaches in *Drosophila* are being used to address these issues.

I. Mechanisms of Axonal Guidance in *Drosophila*.

A. *Glial pathways.* The midline glia (which originate from the mesectoderm), longitudinal glia (which originate from a glioblast), and nerve root glia appear to provide both permissive substrates for nerve outgrowth and some level of instructive information for the differential guidance of the initial "pioneering" growth cones as they actively choose which glia to extend toward and along. When these glial cells are eliminated selectively by either laser or genetic ablation, specific pathways do not form, leading to the conclusion that these early glia play an important role in establishing the prepattern for the central nervous system (CNS) scaffold and nerve root axon pathways. Using the "enhancer trap" method, members of the laboratory have developed a variety of molecular lineage markers for these different classes of embryonic glia and have identified and cloned new genes specifically expressed by these glia.

B. *Axon pathways.* Once the initial axon pathways are established, the predominant guidance cue for subsequent follower growth cones is the surface of the earlier axons in these pathways. Growth cones are able to distinguish one axon bundle, or fascicle, out of an array of many such pathways within their filopodial grasp. The analysis of this phenomenon led Dr. Goodman and his colleagues several years ago to propose the hypothesis of labeled pathways, which predicts that the orthogonal array of commissural, longitudinal, and peripheral nerve root pathways are differentially labeled by recognition molecules that allow growth cones to navigate through these complex choice points.

II. Molecular Genetics of *Drosophila* Adhesion Molecules.

Two different methods have been used to identify and clone numerous cell and substrate adhesion molecules in *Drosophila*. In one set of studies, cell and substrate adhesion molecules were identified that are the homologues of well-known vertebrate adhesion molecules (e.g., the three *Drosophila* laminin genes and two different *Drosophila* cadherin genes). In the second set of studies, an immunological approach was used to identify and clone the genes encoding four different surface glycoproteins: fasciclin I, II, and III and neuroglian. These glycoproteins are dynamically expressed on different overlapping subsets of axon fascicles and glia during embryonic development and are thus good candidates for cell adhesion molecules. All four of these proteins appear to be homophilic cell adhesion molecules.

Genetic screens are also presently under way to identify mutations in new genes involved in pathfinding and/or synaptic specificity. Because mutants in several neural cell adhesion molecules (fasciclin I and III) are viable, show no gross CNS defect, but do show behavioral phenotypes, members of the laboratory have begun screens for behavioral mutants (using the P element–mediated enhancer trap method) to identify genes involved in CNS wiring. Dr. Alex Kolodkin has isolated and is genetically and phenotypically characterizing several new mutations that affect flight behavior and are associated with β-galactosidase expression in subsets of developing neurons.

A. *Laminin.* Dr. Denise Montell cloned the genes that encode the three subunits of *Drosophila* laminin, a substrate adhesion molecule shown to be a potent promoter of neurite outgrowth by previous studies on developing vertebrate neurons. Dr. Russell Rydel has isolated a lethal mutation in the gene encoding the A subunit of laminin (*lama*). This mutation appears to be associated with defects in neurite outgrowth and pathfinding in the developing peripheral nervous system, defects in

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the formation of the CNS longitudinal connectives, and defects in muscle development. Dr. Rydel and Dr. Luis Alonso Garcia are currently generating new mutants in *lama*, conducting a mosaic analysis of its function during wing morphogenesis, and searching for mutations in the laminin B2 subunit.

B. *Cadherins.* Dr. Paul Mahoney used the polymerase chain reaction (PCR) method to clone the genes that encode two different cadherins (calcium-dependent cell adhesion molecule) in *Drosophila*, the first cadherins identified outside of the chordates. Both of these *Drosophila* cadherins appear to be E-cadherin–like molecules, in that both are expressed at high levels in the embryonic ectoderm and in larval imaginal disks. With the help of Dr. Peter Bryant (University of California at Irvine), Dr. Mahoney has shown that the first cadherin is encoded by the *fat* gene; mutations in the *fat* gene lead to a hyperplastic, cell proliferation phenotype of imaginal disks. Hilary Clark has been characterizing the second cadherin and has identified candidates for mutations in this gene. Others are searching for additional members of the cadherin family in *Drosophila*, with a focus on identification of cadherins expressed during neuronal development.

C. *Immunoglobulin superfamily* An immunological approach was used to identify four different surface glycoproteins, fasciclin I, II, and III and neuroglian, which are expressed on subsets of axon fascicles and glia during embryonic development. Drs. Allan Harrelson, Allan Bieber, Peter Snow, and Michael Hortsch showed that fasciclin II and neuroglian are members of the immunoglobulin superfamily and are highly related to a series of vertebrate neural cell adhesion molecules, several of which appear to function as homophilic adhesion molecules. Fasciclin II is more highly related to N-CAM, and neuroglian is more highly related to L1. Fasciclin II has five immunoglobulin (Ig)-like domains and two fibronectin (FN) type III domains, whereas neuroglian has five Ig-like domains and four FN type III repeats. Dr. Bieber has identified a lethal, null mutation in the *neuroglian* gene and with Dr. Hortsch is beginning a genetic analysis of its function both *in vivo* and in transfected cell lines.

The *amalgam* gene encodes another Ig superfamily molecule expressed in the developing *Drosophila* nervous system. Since joining Dr. Goodman's laboratory, Dr. Mark Seeger has continued a genetic analysis of *amalgam* function during *Drosophila* development and has shown that the amalgam protein is secreted but yet is found associated with neuronal membranes, suggesting the presence of amalgam receptors and/or binding proteins.

D. *Novel cell adhesion molecules.* The other two proteins (fasciclin I and III) uncovered by the immunological screen for molecules expressed on subsets of axon pathways are unrelated thus far to anything currently in the data bank. To begin to test their function, Drs. Snow and Bieber and Dr. Tom Elkins used molecular genetic techniques to induce the expression of fasciclin on the surface of *Drosophila* S2 cells in cell culture. Using standard aggregation assays and biochemical methods, these workers were able to show that both fasciclin I and III are homophilic adhesion molecules that appear to define new classes of adhesion molecules. Dr. Elkins has generated viable, null mutations in the genes encoding both of these novel neural cell adhesion molecules and is conducting a detailed genetic analysis of their function. Studies in progress (in collaboration with Dr. Martin Heisenberg in Germany) suggest that both mutants may have interesting behavioral phenotypes: *fasciclin I* mutants are uncoordinated in their leg movements, whereas *fasciclin III* mutants appear to have abnormal optimotor orientation and responses.

E. *Involvement of cell adhesion molecules in signal transduction.* To begin to unravel the potential interactions of neural cell adhesion molecules with different second messenger systems, Dr. Elkins has examined the phenotypes of embryos doubly mutant for one of several adhesion molecules (*fasciclin I*, *fasciclin III*, or *neuroglian*) and one of two tyrosine kinases known to be expressed at high levels in developing neurons (the *Drosophila* homologues of the *src* and *abl* cellular oncogenes). In collaboration with Dr. Michael Hoffman (University of Wisconsin), Dr. Elkins has discovered a powerful interaction between *fasciclin I* and *abl*. Whereas at a gross level the CNS develops relatively normally in either *fasciclin I* or *abl* mutant embryos, in double mutants, CNS development is highly abnormal. Cell determination and initial axon outgrowth appear unaffected. However, they fail to make the proper pathway choices; the major outcome is that the two large commissural pathways in each segment do not form, and the longitudinal connectives are often abnormal.

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Dr. Goodman is also Class of '33 Professor of Genetics in the Department of Molecular and Cell Biology at the University of California at Berkeley and Adjunct Professor in the Department of Physiology at the University of California School of Medicine at 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, Dr. Horvitz and his colleagues have isolated developmental mutants of the nematode *Caenorhabditis elegans* and have used genetic and molecular genetic techniques to characterize these mutants. 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. In this way, genes involved in cell lineage, cell death, cell migration, and cell differentiation have been identified and analyzed.

I. 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 one major focus of the research of this laboratory. Hundreds of cell lineage mutations have been identified that cause certain cells to express lineages or fates normally associated with certain other cells. For example, a mutation in the newly identified gene *lin-44* causes certain sister cells that normally differ in their fates to be reversed in their fates. Mutations in the gene *lin-11* cause certain sister cells that normally differ in their fates to express the same fate. The DNA sequence of *lin-11* suggests that the *lin-11* protein contains two DNA-binding domains (a homeodomain and a zinc-binding domain) as well as a domain involved in protein-protein interactions (a proline-rich domain). Mutations in the gene *unc-86* cause certain daughter cells to express the fates of their mother cells. The DNA sequence of *unc-86* reveals that the *unc-86* protein is strikingly similar over a region of ~160 amino acids to three mammalian transcription factors (Pit-1 and the octamer-binding proteins Oct-1 and Oct-2), which has led to the name of the common POU motif (*pit*, *oct*, *unc*) and to the suggestion that these mammalian genes not only regulate gene expression but also specify cell fates during development.

The cell lineages of vulval development, which are determined via cell-cell interactions, have been analyzed in some detail. A genetic pathway for vulval development that now consists of 46 genes has been defined. Most of these genes participate in the determination of the fates of the vulval precursor cells. Of the determination genes, *lin-10* has been studied most extensively. Combined molecular and genetic analyses have indicated that this gene encodes a 45 kDa polypeptide that acts within the cytoplasm of the vulval precursor cells to transduce information from a membrane receptor to the nucleus. Despite the highly limited temporal and spatial localization of *lin-10* function as defined genetically, *lin-10* mRNA and protein are expressed very broadly. It is possible that it is the specificity of expression of other genes involved in vulval development that leads to the specificity of function of *lin-10*.

II. 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. Six additional genes (*ced-1*, *-2*, *-5*, *-6*, *-7*, and *-8*), four of which are newly discovered, are necessary for the corpse of a cell undergoing programmed cell death to be engulfed by a neighboring cell. Analysis of these genes may reveal the molecular mechanisms responsible for this specific cell-cell interaction. A number of mutations have been identified that cause cells to die that normally survive. Some of these mutations cause the ectopic activation of *ced-3* and *ced-4*, whereas others result in the cytotoxic expression of particular gene products. Because cell death is the cause of the major clinical features of human neurodegenerative diseases, human homologues of *C. elegans* cell death genes are being sought. In addition, linkage analysis of familial amyotrophic lateral sclerosis (ALS or Lou Gehrig disease) has been begun in an attempt to initiate molecular genetic studies of human diseases caused by cell death.

III. 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. For example, the two sex myoblast (SM) cells, which are born in

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the posterior body region, move to a central position along the animal's length, near its gonad. Mutations in two genes cause the SMs to terminate their migrations prematurely. These mutations appear to alter an intercellular signaling system that controls SM migration by causing the SMs to be repelled by rather than attracted to a gonadal signal that normally directs the SM migration.

IV. Cell Differentiation.

Genes involved in the differentiation of nerves and muscles have been identified. For example, many mutations have been shown to affect the development of the axon of the serotonergic hermaphrodite-specific neuron (HSN) motor neuron, which innervates the vulval muscles and stimulates egg laying. The HSN axon normally first grows ventrally and then grows anteriorly; a single branch develops from this axon. These different aspects of HSN axonal outgrowth seem to be under separate genetic control: four genes affect ventral growth, seven genes affect anterior growth, and, because the vulva induces HSN branch formation, a variety of genes identified on the basis of their function in vulval development (see above) affect branching. One of the genes involved in anterior HSN axonal outgrowth, *unc-76*, defines a component specifically required for HSN growth within the ventral nerve cord.

Muscle differentiation has been examined by identifying mutants abnormal in muscle structure and function. Five interacting genes involved in the regulation of muscle contraction have been identified. One of these genes (*unc-93*) has been cloned and found to encode a novel protein with multiple putative transmembrane domains. The other four genes known to interact with *unc-93* may define other muscle membrane proteins or may define proteins that regulate the activity of *unc-93*.

Dr. Horvitz is also Professor of Biology at the Massachusetts Institute of Technology and Neurobiologist at Massachusetts General Hospital.

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MOLECULAR MECHANISMS IN THE REGULATION OF NEUROTRANSMITTER RECEPTOR FUNCTION

RICHARD L. HUGANIR, PH.D., *Associate Investigator*

Synaptic transmission is the process by which neurons in the brain communicate with each other through chemical neurotransmitters. During synaptic transmission, an action potential in the presynaptic neuron triggers the release of neurotransmitters from the nerve terminal; these neurotransmitters then diffuse across the synaptic cleft and bind to neurotransmitter receptors in the postsynaptic membrane. The binding of the neurotransmitter to the receptor causes the opening of ion channels in the postsynaptic membrane, allowing ions to diffuse down their electrochemical gradient and depolarize the cell. This depolarization triggers an action potential in the postsynaptic cell and thus completes the process of synaptic transmission.

Dr. Huganir's laboratory is interested in the molecular mechanisms that underlie the regulation of synaptic transmission. Recent studies in many laboratories have provided evidence that protein phosphorylation may be one of the major mechanisms in the control of synaptic transmission. To analyze the role of protein phosphorylation in the control of synaptic function, Dr. Huganir and his colleagues have used the best-characterized neurotransmitter receptor in neurobiology, the nicotinic acetylcholine receptor, as a model system. The nicotinic acetylcholine receptor is a chemically gated ion channel that is a pentameric complex of four types of subunits, in the stoichiometry $\alpha_2\beta\gamma\delta$. The nicotinic receptor mediates the response to the neurotransmitter acetylcholine at the postsynaptic membrane of nicotinic cholinergic synapses such as the neuromuscular junction.

I. Characterization of Protein Phosphorylation of the Nicotinic Acetylcholine Receptor.

Initially Dr. Huganir and his colleagues examined the role of protein phosphorylation in the regulation of synaptic transmission at nicotinic synapses by characterizing the protein kinases that phosphorylate the nicotinic receptor. Postsynaptic membranes highly enriched in the nicotinic receptor were isolated from the electric organ of *Torpedo californica* and were found to contain at least three different protein kinases that phosphorylated the nicotinic receptor. The cAMP-dependent protein kinase phosphorylated the receptor on the γ- and δ-subunits, protein kinase C phosphorylated the δ- and α-subunits, and a protein tyrosine kinase activity phosphorylated the β-, γ-, and δ-subunits of the receptor. Each of these kinases phosphorylated a unique site on each subunit; thus three different protein kinases phosphorylated the receptor on a total of seven distinct sites. All seven of these phosphorylation sites are located on a homologous region on the major intracellular loop of each subunit. In addition, multiple phosphorylation sites on a single subunit are clustered together; for example, the three phosphorylation sites on the δ-subunit are located within 20 amino acids of each other, suggesting that phosphorylation of the receptor by all three protein kinases regulates a common property of the receptor. All of these phosphorylation sites have been found to be conserved in the amino acid sequence of acetylcholine receptor subunits from all species sequenced so far, except for the phosphorylation sites on the γ-subunits from mammalian species. However, the cAMP-dependent phosphorylation site is conserved in the adult form of the γ-subunit (ε-subunit) in mammalian species.

To identify the protein tyrosine kinase(s) that phosphorylates the receptor, Dr. Huganir and his colleagues are using protein purification and molecular cloning techniques. Immunological cross-reaction with known protein tyrosine kinases and partial purification of the protein tyrosine kinases have shown that there are at least two distinct protein tyrosine kinases in *Torpedo* electroplax. The 60 kDa protein tyrosine kinase is related to $pp60^{csrc}$, a well-characterized protein tyrosine kinase. The other protein tyrosine kinase appears to be a novel 90 kDa protein kinase that seems to be specific to the acetylcholine receptor-rich postsynaptic membranes. The 90 kDa protein has recently been partially purified, and attempts are being made to obtain the amino acid sequence of the protein to prepare specific oligonucleotide probes for cDNA cloning. In addition, polymerase chain reaction and cDNA cloning techniques have been used to identify four different protein tyrosine kinases that are expressed in the *Torpedo* electric organ. These four different protein tyrosine kinases are being analyzed to determine which of them is involved in the phosphorylation and regulation of the nicotinic acetylcholine receptor.

The nicotinic acetylcholine receptor is phosphorylated in muscle cells by the same protein kinases

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that were observed to phosphorylate the receptor in isolated postsynaptic membranes from *Torpedo*. Agents that activate cAMP-dependent protein kinase (such as forskolin and cAMP analogues) or agents that activate protein kinase C (such as phorbol esters) have been shown to regulate the phosphorylation of the nicotinic receptor in primary muscle cell cultures. In contrast, although the receptor is phosphorylated on tyrosine residues in muscle cells, it is phosphorylated to a very low level, and it is not known how this phosphorylation is regulated.

Dr. Huganir and his colleagues have used muscle cell cultures to investigate the neurotransmitters, hormones, and neuropeptides that modulate nicotinic acetylcholine receptor phosphorylation. Recently a neuropeptide, calcitonin gene-related peptide (CGRP), has been found to be a cotransmitter with acetylcholine at the neuromuscular junction. CGRP regulates the phosphorylation of the acetylcholine receptor in a manner identical to that of agents that activate cAMP-dependent protein kinase. These results suggest that CGRP regulates acetylcholine receptor phosphorylation by the activation of cAMP-dependent protein kinase and that CGRP may be a physiological modulator of nicotinic acetylcholine receptor function at the neuromuscular junction.

Recent studies have suggested that the first messenger that regulates protein kinase C phosphorylation of the acetylcholine receptor may be acetylcholine itself. Acetylcholine has been found to regulate receptor phosphorylation in an identical manner to that of phorbol esters, potent activators of protein kinase C. The molecular mechanism of the activation of protein kinase C by acetylcholine is not yet clear, but it may be that calcium ions that permeate the nicotinic receptor channel directly or indirectly activate protein kinase C.

The first messengers that regulate the tyrosine phosphorylation of the nicotinic receptor are not known. However, recent studies in Dr. Huganir's laboratory have suggested that the presynaptic neuron is intimately involved in the activation of the protein tyrosine kinase. When immunocytochemical techniques with antibodies to phosphotyrosine were used, it was found that in intact rat diaphragm, the nicotinic acetylcholine receptor is highly phosphorylated on tyrosine residues. Moreover, denervation of the muscle leads to a progressive decrease in phosphotyrosine phosphorylation. In addition, during development, tyrosine phosphorylation of the receptor does not occur until after innervation of the muscle. These results strongly suggest that something from the nerve, either a diffusible substance or the physical contact of the nerve terminal itself, activates the tyrosine phosphorylation system.

II. Functional Effects of Protein Phosphorylation of the Nicotinic Acetylcholine Receptor.

To study the functional effects of nicotinic receptor phosphorylation, Dr. Huganir's laboratory has examined how phosphorylation of the receptor alters its ion channel properties. These studies were performed using purified preparations of the receptor reconstituted in phospholipid vesicles. These results demonstrated that phosphorylation of the γ- and δ-subunits of the receptor by cAMP-dependent protein kinase increases the rate of desensitization of the receptor. Desensitization is a common property of all receptors and is the process by which a receptor is reversibly inactivated in the presence of its agonist. Patch-clamp techniques were used to analyze the single-channel properties of the reconstituted receptor phosphorylated on tyrosine residues. The results have demonstrated that tyrosine phosphorylation of the β-, γ-, and δ-subunits of the receptor also increases the rate of desensitization of the receptor and suggest a widespread role for tyrosine phosphorylation in the modulation of synaptic function.

The functional effects of phosphorylation of the receptor are also being studied by site-specific mutagenesis of the phosphorylation sites on the receptor subunits. All of the known phosphorylation sites have been mutated, and the mutant subunits have been expressed in *Xenopus* oocytes. Expression of the mutant subunits produces a fully assembled receptor with a normal subunit stoichiometry of $\alpha_2\beta\gamma\delta$, although the receptor subunits are not phosphorylated. In collaboration with Dr. Gary Yellen (HHMI, The Johns Hopkins University), the mutant receptors have been shown to be functional by using intracellular recording techniques as well as single-channel recording techniques. The desensitization kinetics of the mutant receptors are currently being analyzed and compared with the kinetics of desensitization of normal receptors.

The results from these studies suggest that phosphorylation of the nicotinic acetylcholine receptor by various protein kinases integrates the effects of several convergent regulatory pathways on cholinergic synaptic transmission. Moreover, protein phosphorylation of neurotransmitter receptors and ion channels may be the major molecular mecha-

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nism for the regulation of their function and thus may play a primary role in the regulation of synaptic plasticity.

Dr. Huganir is also Associate Professor of Neuroscience at The Johns Hopkins University School of Medicine.

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SIGNAL TRANSDUCTION MEDIATED BY G PROTEINS

JAMES B. HURLEY, PH.D., *Associate Investigator*

Signal transduction mechanisms mediated by G proteins include responses of cells to light, hormones, neurotransmitters, and chemotactic agents. G proteins transduce extracellular signals into regulation of specific effectors that alter cellular properties. Like the receptors and effectors that they interact with, G proteins are a diverse family encoded by many different closely related genes. Dr. Hurley and his colleagues are studying how the diversity of the G protein family generates a variety of cellular responses.

I. G Proteins and Phototransduction.

Rod and cone photoreceptor cells hyperpolarize when exposed to light. Light acts directly on the chromophore of rhodopsin, retinal. The subsequent response is mediated by transducins, a specific class of G proteins. Activated rhodopsin catalyzes GTP binding to the largest subunit of transducin Tα. This form of transducin then inactivates an inhibitor subunit of a cyclic GMP (cGMP) phosphodiesterase. Cyclic GMP in the photoreceptor cell is then rapidly hydrolyzed, a cGMP-dependent cation channel is shut down, and the cell hyperpolarizes.

This pathway exists in both rod and cone photoreceptor cells. Rods are very sensitive to light, but slow to respond, whereas cones are insensitive to light but quick to respond. Distinct forms of opsins, transducins, and phosphodiesterases have been identified in both cell types. Dr. Hurley and his colleagues have been studying cone transducin and a cone phosphodiesterase inhibitor to determine if properties of these enzymes are responsible for the physiological differences between rods and cones. Cone transducin and cone phosphodiesterase inhibitor cDNA clones have been identified by screening cDNA libraries at low stringency, using rod cDNAs as probes. Antipeptide antibodies were used to show that the proteins encoded by these cDNA clones are found only in cone cells. Anti-opsin probes were also used, together with anticone transducin probes, to demonstrate that cone transducin is present in red-, green-, and blue-sensitive cones in human retinas. Recently, a monoclonal antibody raised against a cone transducin fusion protein has been used to affinity-purify cone transducin from bovine retinas. The purified cone transducin is being used to compare its kinetic properties with those of rod transducin. The epitope recognized by this antibody has been mapped by testing its immunoreactivity against a series of fusion proteins successively deleted from the amino terminus. Dr. Hurley and his colleagues also characterized a cone-specific form of the phosphodiesterase inhibitor subunit. Antipeptide antibodies raised against sequences unique to this protein recognize only a subpopulation of cones in bovine retinas. These cones correspond to the blue-sensitive cones present in human retinas. Dr. Hurley and his colleagues plan to express the rod and cone phosphodiesterase inhibitors in bacteria to compare their properties. A cone-specific phosphodiesterase has been purified in the laboratory of Dr. Joseph Beavo, and Dr. Hurley and his colleagues plan to use all of these proteins to compare the kinetic characteristics of the rod and cone cGMP phototransduction cascades.

II. *Drosophila* G Proteins.

The roles of G proteins in *Drosophila* signal transduction pathways are also being investigated. *Drosophila* G protein α- and β-subunits were identified by isolating *Drosophila* genomic and cDNA clones related to mammalian G proteins. Two *Drosophila* α-subunit genes and a β-subunit gene have been characterized. One of the α-subunits is closely related to mammalian G_i α-subunits, whereas the other is closely related to mammalian G_o. Two forms of *Drosophila* G_o mRNA that are produced by alternative splicing encode proteins that differ only at the amino terminus. There appears to be only one β-subunit gene that has no introns within the coding region.

In situ localization analyses of mRNA corresponding to these genes demonstrate that the G_i α-subunit is expressed primarily in the nurse cells of *Drosophila* ovaries, whereas the G_o α-subunit is expressed primarily in nervous tissues. No G protein α-subunit has yet been identified in *Drosophila* eyes. G protein β-subunit mRNA is expressed primarily in nervous tissue, including brain and eyes.

A variety of antibody probes were used to localize the *Drosophila* G_i α-subunit in *Drosophila* sections. These antibodies react most strongly with nuclei in nurse cells and follicle cells in *Drosophila* ovaries. Only very weak reactivity is seen in other tissues. An antipeptide antibody that recognizes a

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sequence unique to this protein recognizes an ~40 kDa protein that is enriched in preparations of nuclei from female *Drosophila* bodies. Several monoclonal antibodies that recognize the *Drosophila* β-subunit react strongly with *Drosophila* eyes, brain, and thoracic ganglion. In ovaries, these antibodies also react strongly with nuclei of nurse cells and follicle cells.

Dr. Hurley and his colleagues continue to investigate the roles of G proteins in *Drosophila*, using a variety of methods, including transformation, with G protein genes having dominant mutations, and production of G protein null mutants. The role of a G protein in nuclei is of particular interest. Similar methods are also being used to study transducins in mammalian photoreceptors. The mouse rod transducin gene has been isolated, and its promoter is being characterized by testing its ability to express β-galactosidase in rod cells of transgenic mice. Once expression in photoreceptors has been achieved, the effects of dominant mutations on the kinetics and sensitivity of phototransduction are to be determined.

Dr. Hurley is also Assistant Professor of Biochemistry at the University of Washington.

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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 found in most cell types studied in the animal and the plant kingdoms. Different combinations of potassium channels are found in different cells and are involved in a variety of cell functions. 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.

To understand better how potassium channels work and how the tremendous diversity of these channels is generated, one would like to study these channels biochemically. Although the sparsity of these channels has presented a serious problem in their purification, the first potassium channel has been cloned in Dr. Jan's laboratory by taking advantage of *Drosophila* genetics. The initial molecular characterizations have provided some clues to questions concerning diversity and structural elements involved in different channel functions.

I. Alternative Splicing at the *Shaker* Locus Generates Potassium Channel Diversity.

The *Shaker* locus in *Drosophila* gives rise to a number of protein products by alternative splicing. These *Shaker* proteins have different amino- and/or carboxyl-terminal regions but have the same core region, including most of the putative membrane-spanning sequences. When *in vitro* transcribed RNA from each *Shaker* cDNA is injected into *Xenopus* oocytes, it induces inactivating potassium channels (A channels) of distinct kinetic properties. Moreover, the different *Shaker* proteins show different distributions in the central nervous system. Thus the different *Shaker* products are likely to form different subtypes of potassium channels; these subtypes may have different tissue distributions as well as different physiological properties.

II. Structure-Function Analysis of the Potassium Channels Encoded by the *Shaker* Locus.

Like other voltage-gated cation channels, the potassium channels encoded by the *Shaker* locus contain intrinsic voltage sensors that detect the electrical potential across the membrane. These sensors are thought to be displaced by depolarization of the membrane, triggering conformation changes that open the channel. After channel opening, the *Shaker* channels are closed by a specialized inactivation gate that remains shut as long as depolarization persists. To study the molecular basis of channel opening and inactivation, Dr. Jan's colleagues have altered the primary structure of these potassium channel components by site-directed mutagenesis, expressed mutant channels in *Xenopus* oocytes, and characterized the potassium currents electrophysiologically.

The intrinsic voltage sensors are expected to correspond to charged or polarizable amino acids of the ion channel that are located within the membrane field. It has been proposed that the basic amino acids of the S4 sequence, which is found in sodium and calcium channels as well as potassium channels, may function as voltage sensors. Each of these basic residues has been substituted one at a time by either a different basic residue or the neutral residue glutamine. Some of these mutants show altered voltage sensitivity of macroscopic current activation and inactivation, without having obvious effects on other functional properties of the channel, such as potassium selectivity or the rate of recovery from inactivation.

In contrast to the voltage sensors, the inactivation gate may be outside of the membrane, as indicated by previous biophysical studies on sodium channels and potassium channels. For this reason, the effects of mutations of the hydrophilic terminal regions are being tested. Preliminary results indicate that some of these mutations alter the kinetic properties of inactivation.

III. Search for Other Channel Genes.

Comparative studies can often provide valuable clues to structure-function analysis. Using *Shaker* sequences, several groups have obtained other channel sequences from mammals, amphibians, and snails. Most of these sequences are fairly similar (~70% amino acid identity). They reveal sequences that are highly conserved, as well as those, including regularly spaced hydrophobic residues within some of the putative membrane-spanning regions, that seem to tolerate changes readily. Such information is useful in formulating structural models for experimental tests. Detailed comparison of channel function, similar to what is being carried out for the different *Shaker* proteins, may also be

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useful in correlating structural differences with functional variants.

Isolation of channels with much greater divergence in structure and function is of obvious interest. A number of approaches are possible and are being explored. One of the approaches is the isolation of P element–induced mutations that cause behavioral and possibly physiological defects or interact with existing mutations known to affect potassium channels (e.g., *Shaker*). Given the acceleration of genetic and molecular characterizations that is possible with insertional mutants that contain a single P element harboring bacterial plasmid origin of replication, it should be possible to characterize a number of genes in a reasonable period of time and determine from the primary structure if some of these genes encode channel proteins or molecules that affect channel function.

Dr. Lily Y. Jan is also Professor of Physiology and Biochemistry at the University of California at San Francisco.

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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. How is a nervous system organized during 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., first, mutants are isolated that affect neurogenesis, neuronal type, or axonal pathway formation. Identification of the mutations can lead to the isolation of genes important in neuronal development.

A suitable preparation for cellular analysis of neural development in mutant and wild-type animals is needed to undertake a thorough genetic and molecular approach. The embryonic sensory nervous system provided an excellent assay system.

This system has been characterized in detail. The position, identity, and likely function for each individual cell are now known. More recently, Dr. Rolf Bodmer worked out much of the DNA replication patterns and cell lineages of this peripheral sensory nervous system by using the thymidine analogue BrdU. This has provided the groundwork necessary for the isolation and analysis of mutants.

During the last few years, Dr. Jan's laboratory has been engaged in an extensive search for mutants affecting neural development in *Drosophila*. More than half of the genome has been screened, and more than 20 genes that are involved in early neural development have been found. Mutations or deletions of these genes lead to one of the following phenotypes: 1) severe hypertrophy or hypotrophy of the nervous system, 2) deletion or duplication of specific subgroups of neurons, 3) transformation of one neuronal type into another, or 4) misrouting of the axonal pathway.

Several of these mutants are likely to play key roles in the development of the embryonic sensory nervous system. These genes are being analyzed at the molecular level.

I. Genes Required for the Formation of Sensory Organ Precursors.

An undifferentiated ectodermal cell can become either an epidermal cell or a sensory organ precursor. Dr. Jan and his colleagues found a gene, *daughterless* (*da*), that plays a crucial role in determining whether a cell becomes a sensory organ precursor. Deletion of *da* was known previously for its role in sex determination, as studied by Dr. Thomas Cline at Princeton University. In *Drosophila*, sex is determined by the ratio of the number of X chromosomes to the number of autosomes. The gene *Sex lethal* (*Sxl*) seems to play a central role. If *Sxl* is on, it turns on downstream genes, such as *tra* and *tra-2*, resulting in female development. *da* is a positive regulator of *Sxl*; the *da* gene product is supplied by the mother and is required early during embryogenesis to turn on *Sxl*. If the maternal supply of the *da* gene product is greatly reduced or absent, *Sxl* is off. This does not affect male development, because *Sxl* is normally off in male embryos. However, in female progeny of *da* mutant female flies, the lack of *Sxl* function causes abnormal dosage compensation and, as a result, lethality. This explains the "daughterless" phenotype. *da* is required not only for sex determination but also for neurogenesis. This is an example of two seemingly unrelated biological processes that are involved in the function of a particular gene.

Recently, Drs. Michael Caudy and Harald Vaessin have cloned *da*. The sequence of the transcripts predicts a protein product of 710 amino acids. It shares significant sequence similarities with a number of genes [the nuclear oncogene *myc*; the *Drosophila achaete-scute complex* (*AS-C*), which is known to be involved in neuronal determination; and *MyoD1*, which can transform fibroblasts into myoblasts]. Moreover, the region of sequence similarity (the *myc*-similarity region) has been shown to be both necessary and sufficient for the myoblast-transforming ability of *MyoD1*. Thus there is growing evidence that this *myc*-similarity region is an important functional motif shared by many genes involved in specifying cell fate.

More recently, a striking similarity has been found between *da* and a human immunoglobulin enhancer-binding protein (75% identity over 85 amino acids of the *myc*-similarity region and the adjacent L repeat). This conserved region contains the DNA-binding activity of the enhancer-binding protein. By analogy, most likely *da* also functions as a DNA-binding transcription regulator.

How might *da* function in such different processes as neuronal development and sex determination? One hypothesis is that the same *da* protein,

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acting as a DNA-binding transcription regulator, is a common factor required for both processes. It may act in conjunction with one set of factors (including, for example, *sisterless B*, a numerator element in the X/A ratio) to initiate sex determination of somatic cells and with another set of factors (including, for example, *AS-C* genes) to initiate neuronal precursor formation. In collaboration with Dr. David Baltimore (Massachusetts Institute of Technology), biochemical evidence has been obtained suggesting the *da* and *AS-C* products may interact directly with each other in regulating transcription of downstream genes.

II. Genes Affecting Neurogenesis.

One major group of mutations affecting neurogenesis was previously identified by Dr. Jose Campos-Ortega and others. This group includes at least seven genes: *Notch* (*N*), *Delta* (*Dl*), *Enhancer of split* [*E(spl)*], *almondex* (*amx*), *mastermind* (*mam*), *neuralized* (*neu*), and *big brain* (*bib*). Deletion of any of these genes leads to hypertrophy of both the central and peripheral nervous systems. Several genes of this group have already been cloned [*Dl*, *E(spl)*, and *N*]. The structure of the gene products suggests a mode of action through cell-cell interaction. Previous genetic and cell transplantation experiments by Dr. Campos-Ortega and his colleagues indicated that those seven genes can be divided into several pathways. According to this scheme, *neu* and *bib* act at steps separate from the ones that have already been characterized at the molecular level [*N*, *Dl*, *E(spl)*]. Thus, to obtain a more complete understanding of neurogenesis, it is desirable to characterize *neu* and *bib* at the molecular level. Dr. Gabrielle Boulianne and Yi Rao have cloned *neu* and *bib*, respectively. The structure of *neu* suggests that it is a DNA-binding protein. The predicted *bib* product appears to be a membrane protein with strong homology to bovine lens major intrinsic protein. The products of these genes are being studied.

III. Genes Required in Specifying the Identity of Sensory Organ Cells.

Drosophila embryonic sensory organs can be divided into three major types according to their morphology and possible function: 1) External sensory organs are situated at the surface of the embryo and are exposed to the external environment (e.g., sensory hairs). These organs probably serve as chemoreceptors or mechanoreceptors. 2) The chordotonal organ is tube-shaped and is attached to the body wall at two ends. These organs probably function as stretch receptors. 3) Multiple dendrite neurons are characterized by their extensive dendritic arborization and are probably touch receptors. Dr. Jan and his colleagues are interested in finding out how these sensory organ cells acquire their identity.

A. cut. The *cut* locus is required for external sensory organs to acquire their correct identity. Embryonic lethal mutations at the *cut* locus cause the transformation of external sensory organs into chordotonal organs. More than 200 kilobases (kb) from the *cut* locus have previously been cloned by Dr. Joseph Jack. In collaboration with Dr. Jack, Dr. Karen Blochlinger and Dr. Bodmer have screened for and isolated DNA complementary to embryonic mRNA (cDNA) that hybridizes to the relevant region of the *cut* locus. From overlapping cDNAs, the sequence of 8,217 base pairs (bp) of the *cut* transcript was determined. The predicted primary translation product consists of 2,175 amino acids with an estimated molecular mass of 240 kDa. The predicted *cut* protein contains a homeodomain. The *cut* homeodomain appears to be the most divergent member of the family of homeodomains. Nevertheless, the nine amino acids that are invariant in all previously characterized homeodomains are unaltered in the *cut* homeodomain. Within the predicted *cut* protein there are three internal repeats of 60 amino acids, with 55–68% amino acid identity. These *cut* repeats appear to be unrelated to any previously determined protein structure. Antibodies were generated against peptides from the predicted sequence to determine where the *cut* gene product is expressed. Immunocytochemistry with two of these antisera revealed that the *cut* gene product is expressed in nuclei of cells of external sensory organs but not in the chordotonal organ. This supports the previous conclusion from mosaic studies that the *cut* gene acts autonomously.

That the *cut* gene product is a homeodomain-containing nuclear protein suggests a specific mode of gene function. By analogy to studies of several other homeobox-containing genes, the *cut* gene product is expected to act as a DNA-binding protein that controls the transcription of certain downstream genes responsible for the actual differentiation of the external sensory organ.

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B. numb. A simple external sensory organ precursor divides twice to give rise to four progeny cells: a neuron, a glia, a socket-formatting cell, and a hair-forming cell. Mutations affecting this cell lineage might be expected to alter neuronal number. *numb* is such a mutation. The phenotype of *numb* is the following: 1) The number of the sensory neurons is severely reduced, to ~10% of normal. 2) All four progeny of a sensory organ precursor are present. Thus the loss of sensory neurons is not due to cell death. 3) The socket and hair are often duplicated in a sensory organ. The simplest interpretation of these observations is that *numb* affects the sensory organ lineage in such a way that the cells that normally give rise to neuron and glia are transformed into cells that form socket and hair. This gene has recently been cloned by Dr. Tadashi Uemura in this laboratory. The sequences suggest that the gene product may contain a zinc finger.

IV. Mutations Affecting Axonal Pathways.

A number of mutants were found to cause abnormality in neuronal pathways. Dr. Edward Giniger has started to analyze some of those mutants.

V. Additional Genes Involved in Neural Development.

Several thousands of lines, each one with a single P insertion, have been generated. The P element used in this laboratory was constructed by Dr. Ethan Bier and Kevin Lee and contains mini-*white* (w^+), *lacZ*, and PUC sequences.

The w^+ gene is used as a genetic marker to follow the insertion. It also contains a *lacZ* gene fused in frame with the transposase. Because the transposase has a weak promoter, the expression of *lacZ* depends on the site of insertion (presumably under the influence of a nearby promoter or enhancer elements). By doing an X-Gal reaction, one can ask whether there is an interesting pattern of expression associated with the insertion. This has two useful applications. 1) Useful cell-type-dependent promoters can be isolated. 2) The β-galactosidase expression is dominant. Heterozygous transformant lines showing interesting patterns can be selected for further tests of possible phenotypes associated with the insertion in homozygotes. One can also attempt to delete the region by isolating mutants that have lost the w^+ gene (and probably the entire P element, perhaps with some flanking sequences) and testing for phenotype associated with the deletion. The PUC sequence containing the origin of replication and ampicillin resistance is used for speedy cloning via plasmid rescue. This mutant screening is designed to find mutants affecting various aspects of function and development of the nervous system.

Dr. Yuh Nung Jan is also Professor of Physiology and Biochemistry at the University of California at San Francisco.

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MOLECULAR MECHANISMS OF VERTEBRATE NEURONAL DEVELOPMENT

THOMAS M. JESSELL, PH.D., *Investigator*

Dr. Jessell's laboratory continues to focus on cellular and molecular mechanisms that control the differentiation and early development of the vertebrate nervous system.

I. Cell Differentiation in the Neural Tube.

In vertebrates, the patterning of the central nervous system begins soon after induction of the neural plate. The regional specification of the neural tube is imposed by local cell interactions that regulate cell differentiation. Dr. Jessell and his colleagues have examined the contribution of a group of epithelial cells located at the axial midline of the neural plate, named the floor plate, to cell patterning within the neural tube.

Studies performed in collaboration with Drs. Gregor Eichele and Christina Thaller (Harvard Medical School) have shown that the floor plate is the source of a morphogen that polarizes developing embryonic tissues. Cells of the floor plate, but not other regions of the neural tube, evoke digit reduplications when transplanted into the anterior region of the chick limb bud. This mimics the morphogenetic activity of a region of the limb known as the zone of polarizing activity (ZPA). Evidence indicates that the ZPA morphogen that specifies limb polarity and digit pattern is retinoic acid. These findings suggest that the floor plate is a focal source of retinoic acid within the developing neural tube. In support of this, Dr. Jessell and his collaborators have found that the floor plate possesses a greater capacity to convert the metabolic precursor retinol to retinoic acid than other regions of the spinal cord. The role of the retinoic acid–like morphogen in the floor plate may be to regulate the differentiation of adjacent neuroepithelial cells and impose pattern along the dorsoventral axis of the neural tube.

Dr. Jessell and his colleagues have also shown that at later developmental stages the floor plate releases a diffusible chemotropic factor that orients the growth of developing spinal neurons. Evidence that the floor plate releases a chemotropic factor that guides commissural spinal axons was obtained by coculturing explants of E11 rat floor plate and dorsal neural tube in a collagen gel matrix capable of stabilizing gradients of diffusible factors. Dorsal spinal cord explants grown in the absence of a floor plate target exhibited little or no axon outgrowth. In contrast, in the presence of a floor plate there was profuse axon outgrowth from the dorsal explant oriented toward the floor plate. The axons that extend from dorsal explants appear to derive from commissural neurons, since they express the cell surface glycoprotein TAG-1, which is selectively expressed by commissural axons.

The factor released by the floor plate is selective for commissural axons and does not affect the pattern of growth of association, motor, or sensory axons. Moreover, the induction of commissural axon outgrowth from dorsal explants is highly specific to the floor plate. Other neural and nonneural embryonic tissues, including the remainder of the E11 neural tube and notochord, do not exhibit activity. The action of the floor plate is not mimicked by defined growth factors and may therefore reflect the presence of a novel chemotropic molecule. Studies to characterize this molecule are in progress.

The analysis of axon pathfinding in Danforth short tail mice, which lack a floor plate, provided additional evidence that the floor plate guides commissural axons. In collaboration with Dr. Jane Dodd, commissural axons in affected embryos have been shown to exhibit marked perturbations in axon trajectory at the ventral midline, in the region normally occupied by the floor plate. Commissural axons project out of the spinal cord, forming a supernumerary nerve that exhibits abnormal projections.

These observations provide evidence that the floor plate regulates neuronal differentiation in several ways: 1) by releasing a polarizing morphogen, 2) by releasing a diffusible chemotropic factor, and 3) by acting as an intermediate target involved in contact guidance.

II. Axonal Glycoproteins Involved in Neuronal Recognition.

The molecular basis of pathway selection by developing spinal axons has also been studied. One mechanism of growth cone guidance appears to involve interactions between glycoproteins on the surface of axons and cell surface or matrix molecules in their environment. The 135 kDa axonal glycoprotein TAG-1 that is expressed transiently on subsets of developing axons and may be involved in the initial stages of axonal growth has been identified.

To provide further information on the structure and function of TAG-1, Dr. Jessell and his col-

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leagues isolated cDNA clones encoding this glycoprotein. The deduced protein sequence of TAG-1 indicates that it is a member of the immunoglobulin gene family, closely related in structure to other axonal glycoproteins implicated in neuronal recognition, in particular F11, L1, and N-CAM (neural cell adhesion molecule). TAG-1 possesses a large extracellular region that contains six immunoglobulin-like domains and four domains that are homologous to type III sequences found in the extracellular matrix protein fibronectin. In addition, TAG-1 contains an Arg-Gly-Asp (RGD) sequence that has been identified in many cell surface and extracellular matrix proteins that interact with the integrin family of receptors. These findings suggest that TAG-1 has multiple distinct binding domains. TAG-1 does not possess a transmembrane domain but has a hydrophobic carboxyl-terminal region that is indicative of membrane anchoring via a glycosyl phosphatidylinositol linkage. In support of this, biochemical studies have shown that TAG-1 is released from the surface of neurons by phospholipase C. The isolation of full-length TAG-1 cDNA clones will permit a detailed analysis of the structure and function of this axonal glycoprotein.

III. Isolation and Functional Properties of Neurotransmitter Receptors.

Molecular studies of the serotonin receptor gene family have continued, in collaboration with Dr. Richard Axel (HHMI, Columbia University). Many of the actions of serotonin within the central nervous system, including the control of affective and perceptual states, are mediated by the 5HT2 receptor subtype. A cDNA encoding a rat brain 5HT2 receptor has been isolated by virtue of its homology with the 5HTlc receptor. The 5HT2 receptor is a new member of the family of G protein–linked receptors that span the lipid bilayer seven times. Overall sequence identity between the 5HT2 and 5HT1c receptors is 49%, but identity within the transmembrane domains is 80%. Expression of functional 5HT2 receptors in *Xenopus* oocytes and transfected mouse fibroblasts indicates that, like the 5HTlc receptor, this receptor activates phospholipase C signaling pathways and elevates intracellular Ca^{2+}. These structural and functional similarities indicate that serotonin receptors derive from at least two gene families. The 5HT1c and 5HT2 receptors define one gene family, whereas the 5HT1a receptor has evolved from a distinct adrenergic receptor lineage.

The functional consequences of activating a brain-specific neurotransmitter receptor, the serotonin 5HT1c receptor, in the unnatural environment of a fibroblast have also been examined. Introduction of functional 5HT1c receptors into NIH 3T3 cells results, at high frequency, in the generation of transformed foci. Moreover, the generation and maintenance of transformed foci requires continued activation of the serotonin receptor. The injection of cells derived from transformed foci into nude mice results in the generation of tumors. These findings indicate that the serotonin 5HT1c receptor functions as a proto-oncogene when expressed in NIH 3T3 fibroblasts. Thus the distinction between a neurotransmitter receptor, a growth factor receptor, and an oncogene may depend critically on the cellular environment.

Dr. Jessell is also Associate Professor of Biochemistry and Molecular Biophysics and Member of the Center for Neurobiology and Behavior at the Columbia University College of Physicians and Surgeons.

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CELLULAR BIOPHYSICS AND NEUROENDOCRINE TRANSPORT AND SECRETION

ROBERT G. JOHNSON, JR., M.D., PH.D., *Associate Investigator*

Dr. Johnson is interested in the application of biophysical and molecular techniques with physiological, spatial, and temporal resolution to the investigation of cellular regulation of biological membrane transport and release processes in endocrine secretory cells. Specifically, the laboratory is investigating the cellular regulation and turnover of the Na^+,K^+ ATPase, the control and kinetics of voltage-dependent calcium channels, and the plasticity of chemical messengers in mature secretory vesicles.

I. Cellular Regulation of the Na^+,K^+ ATPase.

The Na^+ pump, which appeared over 600 million years ago, functions to maintain an anisotropic distribution of Na^+ and K^+ across biological membranes by catalyzing the transport of K^+ influx and Na^+ efflux against their respective gradients, using the high-energy phosphogens of ATP. Present in the plasma membranes of most eukaryotic cells, the Na^+ pump has important functions in ion homeostasis, volume regulation, Na^+-coupled solute transport, and the excitability of certain cells. The regulation of the turnover of this ubiquitous pump has been thought to be primarily the ambient intracellular sodium concentration. However, a large body of phenomenological evidence suggests that, in certain cells, hormones and intracellular messengers can independently and significantly increase the Na^+ pump activity. It is difficult to measure accurately and quantitatively Na^+ ATPase activity and the intracellular sodium concentration with reasonable kinetic time constants within intact cells. Understanding the cellular regulation of the Na^+ pump has important implications, given the demonstrated derangements in Na^+ pump function in the pathologic states of hypertension and diabetes. Dr. Johnson and his colleagues have attempted to develop model systems and methodologies to measure in a noninvasive and nondestructive manner the Na^+ pump activity in an intact cell or an intact tissue. The choice of an elasmobranch, *Narcine brasiliensis*, for this study has many advantages, including 1) the presence within the electrocyte of the highest density of Na^+ pump found within nature, 2) the homogeneous nature of the electric organ, 3) the simple composition with few proteins within the dorsal membrane, and 4) a primarily glycolytic metabolic pathway.

The noninvasive, nondestructive technique of nuclear magnetic resonance spectroscopy has been used to measure the ATPase activity of the Na^+ pump in a time-dependent fashion; simultaneously the transmembrane sodium gradient has been measured through the use of custom triple-tuned surface coils. The resting ATPase activity is quite low, but with stimulation the activity increases over three orders of magnitude, despite only a small increase in the internal sodium concentration. Other noninvasive techniques, including saturation transfer measurements, have confirmed this finding. These measurements represent the first evidence that the Na^+ pump activity in an excitable tissue can be significantly regulated by a mechanism independent of intracellular sodium. Ongoing studies are focused on the biochemical signals that regulate the activation and deactivation of the Na^+ pump activity.

II. Plasticity of Neuroendocrine Chemical Messengers.

One significant observation during the past decade has been that many neurotransmitters and neuromodulators are colocalized to the same secretory vesicle and that the amount of each compound can be regulated independently. The chromaffin granule from the adrenal medulla has provided a unique opportunity to study the plasticity of chemical messengers, because of the amount and diversity of the compounds contained there (including catecholamines, enkephalins, and ATP). The enkephalins are present primarily in the high-molecular-weight precursor form, but under the appropriate conditions the degree of processing can be increased significantly. Extensive analysis has indicated that enkephalin content and enkephalin processing can increase when catecholamine content decreases. Radiochemical labeling studies support the conclusion that although enkephalin synthesis and processing increase in newly formed granules, there is a significant increase in processing in mature granules. These findings indicate that a transmembrane signal across the secretory vesicle membrane must activate enzymes responsible for this increased processing. The ability to influence processing of peptides within a mature granule has important implications for secretory vesicles within a nerve terminal, where processing could conceiv-

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ably influence the class of neuropeptide ultimately formed.

III. Regulation of Neuroendocrine Secretion.

The release of a hormone or neurotransmitter from the specialized secretory vesicles into which they are transported and stored at high concentration is dependent on a series of biochemical events, which have not been fully elucidated. Initiation of secretion in most cells appears to be involved critically with an increase in the cytosolic calcium concentration arising from the influx of calcium through voltage-sensitive calcium channels and/or release of calcium from internal subcellular organelles. Likewise, the routes of removal of calcium have not been fully ascertained but include intracellular buffering capacity, the plasma membrane, and intracellular organelles. One impediment to solving the secretion paradigm is the extreme speed under which ion fluxes and fusion events proceed under physiological conditions. In many of these systems, secretion occurs over a millisecond time range, with ion fluxes exquisitely controlled by membrane voltage and ion channel conductance. To investigate the mechanism of action of ion-translocating ATPases, ion channels, ion fluxes, and the fusion event, Dr. Johnson and his colleagues have applied and perfected techniques of electrophysiology—digital time-resolved microspectrofluorometry of single cells, fast-sensitive detectors, and rapid perfusion systems. The data indicate the extremely complex regulatory control of secretion. For example, in collaboration with Dr. M. Morad, it has been found that the secretion from the catecholamine-containing chromaffin cell can be modulated by regulation of the acetylcholine receptor, by the frequency of stimulation on the opening of the calcium channel, and by the feedback inhibition of chemical messengers released from the secretory vesicle upon calcium influx. Experiments have also indicated that release of H^+ from the secretory vesicle (where the pH is 5.5) into the extracellular space has a marked localized inhibitory effect on calcium channel conductance.

The continuing application of sensitive and rapid biophysical techniques to the study of the temporal and spatial distribution of ion fluxes and gradients should provide insights into the mechanism of secretion.

Dr. Johnson is also Associate Professor of Medicine, Physiology, and Biochemistry and Biophysics at the University of Pennsylvania School of Medicine and Attending Physician at the Hospital of the University of Pennsylvania.

CELL AND MOLECULAR MECHANISMS OF LEARNING

ERIC R. KANDEL, M.D., *Senior Investigator*

I. Molecular Mechanisms of Long-Term Memory.

In a variety of learning tasks, long-term memory differs from short-term memory in requiring protein synthesis during training. Sensitization of the gill- and siphon-withdrawal reflex in *Aplysia californica* is a simple form of learning in which this requirement can be studied in individual identified sensory and motor neurons that participate in the storage of both the short- and long-term memory. Behavioral training for sensitization in the intact animal or application of serotonin (a transmitter that mediates sensitization) to individual sensory and motor cells in cultures leads to presynaptic facilitation, an enhancement of transmitter release from the sensory neurons. A single tail stimulus or a single application of serotonin (5-HT) or cAMP (the second messenger activated by 5-HT) causes a transient increase in synaptic strength lasting minutes that is independent of new protein and RNA synthesis. In contrast, five tail stimuli or five repeated applications of 5-HT (or cAMP) give rise to long-term enhancement lasting one day or more that requires both translation and transcription.

A. *Injection of the cAMP-responsive element into the nucleus of* Aplysia *sensory neurons blocks long-term facilitation.* To gain insight into the molecular events that lead to long-term presynaptic facilitation in sensory neurons, A. Barzilai, T. E. Kennedy, J. D. Sweatt, and Kandel used analytical two-dimensional gels to analyze 5-HT–stimulated [^{35}S]methionine incorporation into proteins. 5-HT rapidly stimulated transcriptionally dependent changes in 15 early proteins that peaked within 15–30 min and subsided within 1–3 h. Of the early proteins, 10 showed increases and 5 decreases in net incorporation of label. The same 15 early proteins were also induced by cAMP. In these features—rapid and transient induction, transcriptional dependence, and second messenger mediation—these early proteins resemble the immediate early gene products induced in vertebrate cells by growth factors.

Most cAMP-inducible genes so far studied are activated by specific transcription factors that bind to the enhancer sequence TGACGTCA, called the cAMP-responsive element (CRE). The CRE binds as a dimer of 43 kDa protein subunits, the enhancer-binding protein (CREBP). P. Dash, B. Hochner, and Kandel have now found that extracts of *Aplysia* sensory neurons contain proteins that specifically bind to a mammalian (somatostatin) CRE sequence. The *Aplysia* protein resembles the mammalian CREB protein, in that it is ~45 kDa and serves as a substrate for the A kinase. Microinjection of oligonucleotides containing the CRE sequence into the nucleus of a sensory neuron selectively blocks the long-term increase in synaptic strength without affecting short-term facilitation. Injection of control enhancer sequences fails to block the increase in synaptic strength. These results indicate that transcription of cAMP-inducible genes is required for the expression of long-term facilitation.

B. *Acquisition of long-term facilitation in* Aplysia *sensory neurons leads to a novel mechanism for the persistent activation of the A kinase: protein synthesis-dependent loss of regulatory subunits.* One of the consequences of the transcriptionally dependent alterations, produced in the sensory neurons by repeated application of 5-HT, is the persistent phosphorylation of a set of substrates also phosphorylated transiently after one pulse of 5-HT. To explore possible mechanisms underlying sustained phosphorylation, P. Bergold, J. H. Schwartz (HHMI, Columbia University), Sweatt, and Kandel examined changes in the properties of the *Aplysia* cAMP-dependent protein kinase (A kinase), a heterodimer of two regulatory (R) subunits that inhibit two catalytic (C) subunits. The A kinase is activated during both short- and long-term sensitization, and the amount of R is lowered as compared with C in the sensory cells of long-term behaviorally sensitized animals. Bergold, Schwartz, Sweatt, and Kandel found that facilitatory stimuli (5-HT or cAMP) also diminish the R-to-C ratio in the sensory neurons and that this reduction in R requires new protein synthesis. Thus one of the functions of the macromolecular synthesis required for long-term facilitation is to synthesize proteins that regulate the cAMP-dependent kinase in a long-term manner. This long-term regulation may account for the persistent increase in protein phosphorylation observed in long-term facilitation.

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II. Long-Term Changes in the Morphology of *Aplysia* Sensory Neurons *In Vitro* Due to 5-HT Resemble Developmental Growth in Being Regulated by the Postsynaptic Motor Neuron.

Process formation of *Aplysia* sensory neurons in dissociated cell culture is regulated by the presence of an appropriate target motor neuron. Sensory neurons cocultured with the identified motor neuron L7 have a more complex morphology than do those cultured alone. Moreover, the motor neurons not only stimulate but also appear to guide the outgrowth of processes from the sensory neurons. D. L. Glanzman, Schacher, and Kandel have now found that the postsynaptic cell is also required for long-term morphological changes in sensory neurons in response to a modulatory transmitter, 5-HT. Application of 5-HT produced an increase in the number of varicosities on the neurites of sensory neurons, but only when they are cocultured with motor neurons. This increase is restricted to neurites that contacted the major processes of motor neurons, suggesting that some signal from the postsynaptic motor cell regulates long-term changes in the morphology of sensory neurons, both during development and with learning and memory.

III. Cloning of *Aplysia* Potassium Channel with Homology to *Drosophila Shaker*.

One of the substrate proteins modulated by 5-HT and cAMP during both short- and long-term sensitization is the S-type K^+ channel. A K^+ channel from *Aplysia* that has high homology to the *Drosophila Shaker*, mouse, and rat K^+ channels has been cloned, to begin the examination of this modulation on the molecular level. Homology screening was performed by P. Pfaffinger, B. Zhao, M. Knapp, J.-F. Brunet, D. Dugan, and Kandel, using the polymerase chain reaction (PCR) on *Aplysia* total nervous system RNA. PCR amplified a single DNA band of 180 bp, the predicted size based on the other K^+ channel clones. This fragment was sequenced and showed >85% amino acid identity to other K^+ channel clones. Using PCR and specific oligonucleotides to this *Aplysia* K^+ channel, Pfaffinger, Zhao, Knapp, Brunet, Dugan, and Kandel cloned the complete coding region. They have identified five different 3′ ends, one of which contains a possible A kinase phosphorylation site. These 3′ ends apparently are generated by alternative splicing, since Southern analysis suggests this is a single-copy gene. The domain containing the transmembrane-spanning regions has >70% amino acid identity to other channels. Efforts to clone the 5′ end are now under way. In addition, Zhao, Kandel, and Pfaffinger are now examining the relationship between the expression of different K^+ channel isoforms and the electrical properties of specific identified neurons.

Dr. Kandel is also University Professor, Center for Neurobiology and Behavior, Departments of Physiology and Psychiatry, Columbia University College of Physicians and Surgeons, located at the New York Psychiatric Institute.

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NEURODEVELOPMENT IN *DROSOPHILA*

FLORA KATZ, PH.D., *Assistant Investigator*

Dr. Katz is interested in the mechanisms by which cell surface interactions in the nervous system govern differentiation and the establishment of neural networks.

The *nac* (*neurally altered carbohydrate*) mutation in *Drosophila* alters a neural-specific glycan carried by multiple cell surface neuronal proteins. The *nac* glycan, which was originally identified as an epitope that is expressed on growth cones and their target cells, was highly correlated with growth cone pathfinding activity in the grasshopper. A biochemical analysis revealed that the epitope was imitated by a common asparagine-linked glycan structure found in plants, which contains unusual xylose and fucose linkages. Lectins and antibodies generated against plant glycoproteins were used to analyze the nature of this structure in *Drosophila*. Both xylose-containing and fucose-containing glycans appear to be present and highly enriched in the nervous system of wild-type flies, but absent in *nac*. The expression patterns (stable and specific expression in the nervous system; transient expression during development in other cell types), evolutionary conservation, and inclusion on multiple proteins suggested that the presence of this carbohydrate might be more important as a marker of neuronal cell surfaces than as a functional unit for the individual proteins that carried the modification and might serve as one model for the extensive use of tissue-specific glycosylation throughout the animal kingdom.

An extensive phenotypic analysis has been carried out. *nac* is a maternal-effect cold-sensitive lethal mutation that arrests early in embryonic development. Although it is 100% penetrant, it shows some variability in the time of arrest, and a small number of animals persist through hatching. Using fluorescently labeled phalloidin [which binds to F-actin and clearly highlights the structure of the embryonic central nervous system (CNS)], Dr. Katz and her colleagues studied the morphology of the CNS in the embryos that achieve neuralization. Although these embryos were often arrested early in the process of creating the commissural network that forms the backbone of the CNS, they were grossly normal in structure until the time of arrest. It is not known whether the arrest is caused directly by altered glycan structure or is a secondary consequence. To assess whether the *nac* alteration is acting as a signal in neural assembly in conjunction with other redundant clues, Dr. Katz and her colleagues constructed double mutants with other candidate mutants that affect neural cell surface proteins; these proteins have mild effects on CNS structure but may cause concerted disruptions with *nac*.

The zygotic cold-sensitive effect of *nac* is to alter the assembly of the eye facets and the morphology of the wing. The eye is mildly altered in both the identities and the relationships of its cells. However, when maternal-effect embryos are allowed to begin development at the permissive temperature and then shifted to the nonpermissive temperature, severe and extensive alterations of the eye occur. This suggests that maternal product contributes to imaginal differentiation.

To explore further the role of the glycan in these phenotypes, Dr. Katz and her colleagues have developed a tissue culture system. Cells derived from dissociated cellular blastoderm embryos can be grown in defined media and survive for long periods of time. Extensive differentiation of these cells into recognizable neural and myotube-like structures occurs within 24 h, which correlates with the cessation of mitosis, as judged by [^{3}H]thymidine uptake experiments. Conditions can be adjusted to prevent this differentiation indefinitely in normal cells and prolong the period of mitosis. This system is useful to study the cellular phenotype of *nac*, both with regard to its ability to differentiate neurons and to sustain mitosis. It also provides an *in vitro* system to study cellular neural interactions.

An ELISA assay has been used to search for additional alleles of *nac* and other members of neural glycan pathways, so far unsuccessfully. In the absence of additional alleles, small deficiencies were induced in the mapped area by γ-irradiation of a marked P element that maps close to *nac*. Some of the 10 new deficiencies uncover *nac*; some do not. These deficiencies, along with other recombinants recovered during meiotic mapping, have been used for a cosegregation analysis to demonstrate that all the phenotypes of the mutant segregate together and hence constitute a single mutation.

A molecular analysis of *nac* is in progress. The mapped area was entered from a clone provided by Dr. Richard Garber (Seattle). As glycan expression is recovered in *nac* maternal-effect embryos injected with wild-type cytoplasm, maternal RNA hybrid selection is being used as a transient assay for the

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identification of the appropriate clone, in conjunction with P element rescue using cosmid DNA-bearing P element ends (from a library provided by Dr. John Tamkun). Ultimately, rescue of all phenotypes by a single transcript should confirm the cosegregation analysis.

Work has also begun on the development of a paradigm to study neural target recognition in the visual system of *Drosophila*. Starting with a Y maze that requires flies to choose between wavelengths of light, flies with disrupted vision can be selected; this screen was successfully used by Dr. Seymour Benzer many years ago and has been used regularly since then by other workers. Among this group of mutant flies will be those unable to make connections in the optic lobe. These connections can be clearly visualized with a whole-mount backfill technique developed in the laboratory. In preparation for a mutant screen, isogenic stocks with strong visual behavior are under construction.

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

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MOLECULAR MECHANISMS UNDERLYING CHEMICAL COMMUNICATION AND TISSUE-SPECIFIC snRNPS

MICHAEL R. LERNER, M.D., PH.D., *Assistant Investigator*

Sex pheromones trigger mating behavior in many members of the animal kingdom. For this activity to occur, three criteria must be satisfied. Pheromone molecules must get to receptors, they must interact with receptors, and they must be inactivated. The sensitive and accurate sensory system specializing in this process is being studied in the moth *Manduca sexta*.

Each antenna from a male moth has ~45,000 sensilla, which are lacking in females. Wax-filled tubules that originate at pores on the chitin-covered surface of the sensilla serve as conduits to the interior. Inside, the receptor lymph fluid bathes and protects the cilia of pheromone-sensitive receptor neurons. However, while the lymph effectively shields the delicate cilia from the air and desiccation, it simultaneously poses an aqueous barrier to the extremely hydrophobic pheromone molecules. The backbone of a typical pheromone is an unbranched chain of ~15–16 carbon atoms, and those for *M. sexta* are no exceptions. The task of solubilization apparently falls on the small pheromone-binding protein (PBP), which, at a concentration of >10 mM, is by far the most abundant protein in the receptor lymph. The onset of synthesis of PBP, the final fall in pupal ecdysone levels, and the ability of antennae to respond electrically to pheromone occur at the same time. *In vitro*, the expression of PBP can be blocked by exposing an antenna to ecdysone.

Pheromone receptors on dendritic cilia serve not only to detect minuscule amounts of particular molecules but also to discriminate between closely related ones. After PBP has transported pheromone across the aqueous lymph, these specific receptors are activated. A 68 kDa membrane protein that may be a pheromone receptor has been identified in the moth *Antheraea polyphemus*. Dr. Lerner and his colleagues are working to clone cDNA encoding this protein.

Once pheromone molecules solubilized in the receptor lymph have interacted with receptors, they must be prevented from contributing background noise by repeatedly stimulating the receptors. All of the pheromones for *M. sexta* are aldehydes, and an aldehyde oxidase (AOX) has been found in the receptor lymph. The time course of its expression is the same as that for the PBP. As a result of the AOX, the half-life of pheromone in sensilla is estimated at 0.6 ms.

One way to control gene expression, particularly in the central nervous system (CNS), is by alternative pre-mRNA processing. The components of the small nuclear ribonucleoproteins (snRNPs) that are involved in splicing are essentially constant from one tissue to the next. Each snRNP particle is composed of a small nuclear RNA (snRNA), or in one case two snRNAs, complexed with at least six common polypeptides: B, D, D′, E, F, and G. There are also several proteins that are specific to certain snRNPs but not to specific cell types. Although the mechanisms responsible for alternative pre-mRNA processing are not well understood, one scenario invokes the existence of snRNPs that could direct tissue-specific RNA-splicing choices. A snRNP fitting this description might have one or more components that are expressed in a tissue-specific manner.

A tissue-specific snRNP polypeptide called N has been identified and cloned. Although expressed primarily in brain, its tissue and cell-type distribution closely correlates with the ability of tissues and cells to make mRNA for CGRP (calcitonin gene-related peptide) from the calcitonin/CGRP gene. N is closely related to B. Conceivably snRNPs containing N in place of B recognize distinct nucleotide sequences in pre-mRNAs and aid in the selection of alternative splice sites.

Another interesting property of N is that, like B, it bears at least one Sm epitope. Besides being the highly conserved epitope common to all U snRNPs, the presence of circulating antibody to Sm is pathognomonic for the autoimmune disease systemic lupus erythematosus (SLE). A specific peptide within the N protein has been found to contain an Sm epitope, as it is recognized by anti-Sm sera from patients with SLE.

Dr. Lerner is also Assistant Professor of Molecular Neurobiology at Yale University School of Medicine.

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SIGNAL TRANSDUCTION AND NEUROGENESIS 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 receptor neurons responsible for this initial step in olfactory signal transduction are unique in their capacity to be replaced continually from a population of neuroblast-like precursors throughout adult life. Dr. Reed is investigating two central problems in neurobiology: How do neurons transduce a complex external stimulus into an electrical signal? What are the molecular processes that accompany neural development?

The initial events in olfactory signal transduction occur in a complex sensory organ, the nose. Molecules that comprise the chemical stimulus we perceive as odor 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. Axons extend from the cell bodies located in the epithelium to glomerular tufts in the olfactory bulb.

The replacement of olfactory neurons from neuroblast precursors occurs continually in adult animals. Acute injury to the olfactory bulb or the receptor neurons leads to the rapid loss of these sensory cells and their subsequent, synchronous replacement.

I. 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. Recently, Dr. Reed has identified several components in the presumptive pathway for olfaction. The laboratory 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 adenylate cyclase activity and has proven 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 and provide some insight into the similarity of olfactory receptors to other known G protein–coupled receptors. The membrane-bound receptors that couple to G proteins share considerable structural similarities. Dr. Reed and his colleagues are attempting to identify olfactory receptors by exploiting these similarities through a variety of molecular cloning approaches.

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 of these forms appears to be 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 and is likely to represent a specialized form of adenylyl cyclase evolved for olfactory signal transduction.

Elucidation of a function for the variety of forms of adenylyl cyclase expressed in mammalian tissues may prove difficult. Dr. Reed's laboratory has therefore 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*. The study of the molecular defect in this mutant may expand understanding of the mechanism of memory processes.

II. Neuron Differentiation and Development.

The olfactory neuroepithelium retains the ability to replace sensory neurons continually in adult animals. This regeneration capacity provides an ideal opportunity to study the process of neuron differentiation and development. Dr. Reed and his col-

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leagues are continuing the characterization of gene products expressed exclusively in this developmental pathway.

The complete understanding of many aspects of neurogenesis in the olfactory system would be greatly aided by the availability of cell lines capable of displaying these differentiation processes in culture. Transgenic mouse lines carrying the SV40 T antigen oncogene under the control of an olfactory neuron–specific promoter have been generated. These animals develop neuroblastomas in the olfactory region. Brian Largent has successfully generated cell lines from these tumors; some of these express neuron-specific gene products that Dr. Reed and his colleagues had previously identified. Presently the laboratory is attempting to expand the repertoire of mature neuronal markers expressed by these cells by altering the cell culture conditions.

Dr. Reed is also Associate 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 and his colleagues have continued to examine molecules in the extracellular environment of neurons that direct their development.

I. Neurotrophic Factors.

Work in Dr. Reichardt's laboratory has focused on nerve growth factor (NGF). In previous studies, Dr. Reichardt showed that target organs of sympathetic and sensory neurons, neurons that are regulated by NGF, express the NGF gene at low, but biologically significant, levels. NGF mRNA and protein were also detected in the central nervous system (CNS), particularly in the target areas of cholinergic neurons in the basal forebrain.

To study the role of NGF and its receptor in early embryonic development, where there are indications that NGF may affect cell determination and axon guidance, Dr. Reichardt and his colleagues have spent the past year studying expression of both the NGF and NGF receptor genes in the developing chick embryo. A baculovirus expression system has been used to express the extracellular domains of both the chick and rat NGF receptors. These truncated proteins have been purified and used to prepare antibodies that interfere with receptor-ligand interactions. These antibodies are being used to study receptor distribution and the consequences of inhibiting receptor function during early development of the avian embryo.

II. Neuronal Adhesion and Process Extension on Extracellular Matrix Constituents.

Work in previous years has indicated that cell surface and extracellular matrix (ECM) constituents are important in regulation of the extent and direction of axon growth *in vitro*. Several of these ECM and cell surface glycoproteins have been shown to be expressed in embryos at positions appropriate for influencing neuronal growth cone behavior. The glycoprotein laminin (LN), one of the ECM constituents in the targets innervated by the peripheral nervous system, has dramatic stimulatory effects on neuronal survival, process outgrowth, and expression of neurotransmitters. The primary receptors used by neurons to interact with LN are members of the integrin superfamily of cell surface heterodimers. One integrin heterodimer with an α-subunit of M_r 180,000 and β_1-subunit of M_r 120,000 was previously purified and shown to be an LN receptor. In the past year, Dr. Michael Ignatius and Laura Goetzl showed that this purified integrin α/β_1 heterodimer functions as a dual receptor for LN and collagen, but not fibronectin (FN). Clones encoding this receptor α-subunit are being characterized.

In previous years, PC12 cells have been used as a neuronal model to study interactions with ECM constituents. These cells were shown to express two integrin α/β_1 heterodimers with α-subunits of M_r 180,000 and 140,000. The immunopurified integrin heterodimers were shown to bind LN and collagen strongly and FN only weakly, mimicking the behavior of the PC12 cells. In the past year, the ligands of the individual integrin α/β_1 heterodimers have been identified. In collaboration with Drs. David Turner (State University of New York at Buffalo) and Salvatore Carbonetto (McGill University), Drs. Kevin Tomaselli and Deborah Hall were able to show that the larger heterodimer is the rat homologue of α_1/β_1. This heterodimer binds to collagen and to the E1-4 fragment of LN (a fragment containing most of the three short arms of the cruciform structure of LN). PC12 cells also were shown to interact with a distinct site in the long arm of LN, using a different integrin. Dr. Tomaselli showed that the second α-subunit expressed by PC12 cells is α_3. Integrin α_3/β_1 heterodimers have been shown by Drs. Kurt Gehlsen and Erkki Ruoslahti to function as LN and FN receptors, binding LN in the long arm of the LN cruciform structure (isolated as fragment E8). In summary, this work showed that PC12 cells interact with two distinct sites on LN. Integrin α_1/β_1 heterodimers mediate binding to LN fragment E1-4 and collagens; integrin α_3/β_1 heterodimers appear to mediate binding to LN fragment E8 and the weak observed binding to FN. These two receptors can account for all observed interactions of PC12 cells with the ECM.

An additional LN-binding integrin α/β_1 heterodimer has been characterized by Dr. Hall in collaboration with Drs. Caroline Damsky (University of California at San Francisco) and Arnoud Sonnenberg (Amsterdam). With a human cell line that does not express the integrin α_3/β_1 heterodimer it was possible to show that these cells also interact with at least two sites on LN. Interactions with the E8 fragment of LN are mediated by integrin α_6/β_1 heterodimers; interactions with the E1-4 fragment

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of LN are mediated by integrin α_1/β_1 heterodimers. Preliminary evidence indicates that integrin α_6/β_1 heterodimers may also be able to bind to the E1-4 fragment. To summarize these results, there now appear to be at least three integrin α/β_1 heterodimers capable of binding to LN.

Individual integrin α/β_1 heterodimers have been identified as receptors for LN, FN, and the collagens. There is also evidence that heterodimers in the β_1 family recognize additional ligands, such as tenascin and vitronectin, for which heterodimeric receptors have not been identified. Dr. Blaise Bossy and Karla Neugebauer have tried to identify these additional receptors. They have shown that the integrin α-subunit, α_{VN}, previously shown to associate with β_3 and β_x, can also associate with β_1. The ligand-binding properties of the α_{VN}/β_1 are being investigated. Dr. Bossy has also sequenced cDNAs encoding a novel integrin α-subunit. Using antibodies prepared to peptides in the sequence of this novel integrin, he has shown that it is an α-subunit of M_r 150,000 that associates with the β_1-subunit. The novel heterodimer has now been purified. Its ligand-binding properties are being examined.

In earlier work, the functions of several integrin β_1-receptors were shown to be regulated during development of embryonic chick retinal neurons and ciliary neurons. In particular, target contact appears to downregulate LN-binding integrins. In the past year, Dr. Ivan de Curtis and Dr. Hall have shown that these changes correlate with the loss of individual integrin α-subunits.

III. Neuronal Adhesion and Process Extension on Cellular Substrates.

In earlier work, Dr. Reichardt and his colleagues showed that both ECM glycoproteins and cell adhesion molecules can function as promoters of axon extension. The relative importance of individual molecules depends on both the type and age of the neurons and the particular cellular substrate. On most cells, multiple independent interactions appear to function to promote neurite outgrowth. On Schwann cells, for example, ciliary neurons were shown to extend neurites by using integrin β_1 heterodimers to interact with the ECM proteins assembled on the Schwann cell surface. The most important of these was shown to be an isoform of LN. The same neurons also use N-cadherin on the growth cone to interact with N-cadherin expressed on the surface of the Schwann cells and L1/Ng-CAM on the growth cone to interact with L1/Ng-CAM on the Schwann cell surface. Dr. Frances Lefcort has been examining regulation of these integrins and cell adhesion molecules in developing and denervated peripheral nerve. She has evidence that all of these glycoproteins are regulated by denervation. Most are induced, but N-cadherin is actually downregulated by denervation. Mechanisms of regulation are being explored.

In previous work studying neuronal interactions with astroglia, Dr. Reichardt and his colleagues demonstrated roles for both β_1 and β_3 integrins and several cell adhesion molecules. Results also indicated that additional adhesive interactions were important but were mediated by uncharacterized receptors and ligands. Motivated by these observations, Dr. Gene Napolitano and Kristine Venstrom have begun an effort to identify novel cell adhesion molecules. As the first step in this project, they prepared antibodies to peptides corresponding to sequences found in all characterized cadherins (N, P, and E). In collaboration with Dr. Jack Lilien (University of Wisconsin), these antibodies were shown to recognize each of the characterized cadherins and several additional glycoproteins in the neuroretina with properties consistent with the possibility that they represent novel cadherins. Pursuing this possibility, Dr. Napolitano isolated cDNAs encoding proteins recognized by the cadherin-specific antibodies, one of which has now been shown to encode a novel cadherin. Studies to demonstrate its function and distribution are being pursued.

Dr. Reichardt is also Professor of Physiology and of Biochemistry and Biophysics at the University of California at San Francisco.

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MOLECULAR MECHANISMS OF DEVELOPMENTAL AND REGULATED EXPRESSION OF NEUROENDOCRINE GENES

MICHAEL G. ROSENFELD, M.D., *Investigator*

I. Molecular Mechanisms of Anterior Pituitary Gland Development.

Dr. Rosenfeld's central research focus has been to define further the molecular mechanisms that dictate the developmental and regulated expression of neuroendocrine genes and to apply these principles to analysis of neuronal gene expression. The extensive characterization of the expression of the growth hormone and prolactin genes in related cell types within the anterior pituitary gland provides an excellent model system for study of developmental regulation of gene expression. The anterior pituitary gland is derived from a common primordium originating in Rathke's pouch, an area of mesoderm discontinuity that makes the precise identification of its ectodermal origins uncertain; after proliferation, the five classical specific cell types appear in a stereotypical order during ontogeny, defined on the basis of the trophic hormone that they elaborate. The structurally related prolactin and growth hormone genes are expressed in discrete cell types—lactotrophs and somatotrophs, respectively—with their expression virtually limited to the pituitary gland. The lactotrophs and somatotrophs are the last cell types to appear in the developing rat anterior pituitary. The co-expression of these two genes within the single cells prior to appearance of mature lactotrophs in a subpopulation of mature anterior pituitary cells and in many pituitary adenomas suggested that the prolactin and growth hormone genes are developmentally regulated by related factors. Data from gene-directed ablation studies in transgenic animals are consistent with the notion that lactotroph lineage is derived from presomatotrophs. The molecular basis of pituitary cell phenotype has been approached by characterizing the cis-active elements that are necessary for tissue-specific expression and then isolating the tissue-specific transcription factors that selectively bind to these elements. High levels of cell-specific expression of the rat prolactin gene are dictated by two separate regions, a distal enhancer (–1,830 to –1,530) and a proximal region. Although both regions alone are capable of directing lactotroph-specific physiological levels of expression of fusion genes in transgenic mice, both regions acting in concert are required to give full physiological levels of expression.

DNase I footprinting experiments reveal that the distal and proximal regulating regions contain multiple, related sequences that appear to bind tissue-specific nuclear protein(s); two similar binding sequences are present in the 180 bp of rat growth hormone 5′-flanking sequences that are sufficient to target somatotroph expression in transgenic mice. Mutation of even a single cis-active element in either gene can reduce gene expression by 80–98%.

Binding sites for the tissue-specific transcription factor Pit-1 are A/T-rich sequences related to the sequences that bind octamer transcription factors and homeodomain proteins. Binding of the Pit-1 factor to its cognate sequences provided the basis for its purification, using affinity chromatography and isolation of a Pit-1 clone from rat pituitary and G/C cell cDNA expression libraries. The coding sequence of Pit-1 predicted a 291–amino acid 32,800 Da protein that contained a 60–amino acid sequence with homology to the homeodomains of *Drosophila* regulatory proteins and a second 76– to 78–amino acid region of homology with several other transcriptional or developmental factors, referred to as the POU-specific domain (Pit-1, Oct-1, Oct-2, and *unc-86*). Expression of Pit-1 in a nonpituitary cell line activates expression of both prolactin and growth hormone fusion genes, even at levels of Pit-1 expression <10-fold those in pituitary (G/C) cells. Expression of the cloned Pit-1 structural gene in bacteria generates a 32,900 Da protein that specifically binds to the tissue-specific elements in the distal and proximal regions of the prolactin gene, as well as the growth hormone promoter, and activates *in vitro* transcription of both prolactin and growth hormone genes at equivalent concentrations. These observations raise the question of the roles of Pit-1 and additional factors in dictating pituitary phenotype and cell-specific pituitary gene expression, which is being evaluated using biochemical and genetic approaches.

II. A Large Family of POU Domain Proteins in Mammalian Brain Development.

Five additional mammalian and two *Drosophila* members of the POU domain gene family have been cloned; all of the known POU domain genes are expressed during neural development and exhibit precisely restricted temporal and spatial patterns of

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precisely restricted temporal and spatial patterns of gene expression. In this regard, the POU domain family of transcription factors resemble the developmental patterns of the hierarchy of regulatory genes that are sequentially activated during *Drosophila* development. The correlation between expression of these genes and cortical and sensory neuronal development suggests possible roles for these factors in determining mature neuronal phenotypes. The potential functions of POU domain proteins in activating specific patterns of gene expression characteristic of mature neurons are under investigation.

III. Hormonal Regulation of Gene Expression.

Growth and regulation of eukaryotes are under complex control and are regulated by diverse families of peptides, collectively referred to as growth factors. The epidermal growth factor (EGF) receptor is a 170 kDa transmembrane glycoprotein with intrinsic protein tyrosine kinase activity. The receptor autophosphorylates multiple sites on its carboxyl terminus and exhibits heterologous regulation due to phosphorylation by protein kinase C. Binding of ligand to the EGF receptor initiates a series of immediate, rapid, and delayed effects that culminate in DNA replication and cell division, including activation of the intrinsic protein kinase activity, increase in intracellular calcium, alterations in intracellular pH, receptor internalization and downregulatory activation of specific gene transcription, and, ultimately, DNA replication and mitosis. The cloning of the EGF receptor has permitted definition of a series of distinct domains, including a carboxyl-terminal inhibitory domain, a distinct region containing an 18–amino acid, acidic helix bounded by turns, that is required for coupling receptor to effective internalization and calcium regulatory mechanisms. The identification of this putative domain and the creation of a series of mutants eliminating its activity permit a critical test of various models of receptor activation increases of intracellular calcium levels. Because a point mutation of the ATP-binding site that eliminates all intrinsic protein kinase activity abolishes all of the known effects of EGF (including receptor internalization), internalization of the receptor itself could represent the critical signaling event. An analysis of a series of mutant receptors has shown that a kinase-active, internalization-defective receptor can transmit signals for regulated gene expression and cell growth, supporting the hypothesis that phosphorylation of specific substrates by this membrane-bound, uninternalized receptor signals the mitotic effects of EGF. Receptor downregulation serves a critical function in preventing signal attenuation, resulting in morphological transformation.

Pit-1 cis-active elements transfer regulation by EGF, thyrotropin-releasing hormone (TRH), and phorbol esters; additional regulatory elements in the prolactin and growth hormone genes have been identified. A second pathway of regulation by calcium, involving type II calcium, calmodulin (CaM)-dependent protein kinase, has been identified based on cloning of the brain-specific CaM protein kinase α and construction of a constitutive variant.

The cloning of ligand-dependent transcription factors and identification of estrogen and T_3 response elements in the rat prolactin and growth hormone genes, respectively, have permitted definition of receptor domains and a study of the molecular basis for positive and negative regulation of gene transcription. Specific classes of T_3 receptors can bind to estrogen response elements with high affinity, but in a transcriptionally inactive form, resulting in a net decrease in gene expression. These data reveal that only a subset of T_3-binding elements function as T_3 response elements. Heterodimers between members of the nuclear receptor superfamily (T_3 receptor and retinoic acid receptor) have been identified and shown to be capable of exerting positive and negative effects on gene transcription, dependent on the sequence of the cis-active sequences to which they bind. The β form of the T_3 receptor forms a heterodimer with the α form of the human retinoic acid receptor, based on critical carboxyl-terminal sequences, exerting opposite transcriptional effects on different T_3 response elements.

IV. Developmental Regulation at a Post-transcriptional Level.

Based on an analysis of the rat and human calcitonin/CGRP (calcitonin gene-related peptide) genes, alternative RNA processing has been demonstrated to represent an important developmental strategy used in the neuroendocrine system to dictate a tissue-specific pattern of polypeptide product production. An analysis of the molecular mechanisms responsible for generating such restricted patterns of gene expression has been initiated, to provide general insights into the molecular strategies critical for development and function of the

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neuroendocrine system. The rat calcitonin/CGRP gene contains six exons; splicing of the first four exons generates calcitonin mRNA, which represents >98% of mature transcripts of this gene in thyroid C cells. In contrast, in the brain and peripheral nervous system, the first three exons are spliced to the fifth and sixth exons, generating the mRNA encoding the precursor of the novel 37–amino acid neuropeptide, CGRP.

The developmental regulation of alternative exon usage in calcitonin/CGRP gene expression is associated with the utilization of alternative poly(A) sites within a single transcription unit. Introduction of the rat calcitonin/CGRP gene under transcriptional regulation of the mouse metallothionein I promoter into fertilized mouse eggs resulted in transgenic animals that expressed calcitonin mRNA as the predominant mature transcript in all tissues not expressing the endogenous calcitonin/CGRP gene, except brain. In the central nervous system, CGRP mRNA was the predominant transcript in many neurons not expressing the endogenous gene. A few neurons produced calcitonin mRNA. These data suggest that calcitonin mRNA is the unregulated choice and that specific RNA processing machinery, predominantly localized to the central nervous system, is required for CGRP mRNA production. A family of snRNPs (small nuclear ribonucleoproteins) expressed in cells capable of generating the CGRP transcript do not appear sufficient to mediate the observed cell-specific RNA-splicing choices. Cell lines that mimicked the behavior of neural and endocrine tissues expressing the endogenous gene have been identified and used for DNA-mediated gene transfer of the unmodified and mutated calcitonin/CGRP gene. These studies have generated evidence supporting a model that predicts that cell-specific differences in splice acceptor choice, mediated by the action of an inhibitory neuronal factor, are the developmentally regulated events that unexpectedly dictate alternative splice acceptor choice.

Dr. Rosenfeld is also Professor of Medicine, Eukaryotic Regulatory Biology Program, University of California School of Medicine at San Diego.

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DEVELOPMENT OF THE *DROSOPHILA* 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. Their current work is focused on the development of the visual system.

I. Development of *Drosophila* Retina.

The compound eye of *Drosophila* is a two-dimensional array of 800 repeating units, or ommatidia. Each ommatidium contains eight photoreceptor cells (R1–R8), as well as pigment cells, lens-secreting cells, and a mechanosensory bristle. Each photoreceptor cell has a distinct cellular identity, based on its position within the ommatidium and its projection pattern to the optic lobes of the brain. The stereotyped arrangement of this small number of nerve cells, together with the dispensability of the visual system under laboratory conditions, makes the compound eye an attractive model system to study genes involved in the specification of nerve cells.

The development of the compound eye of *Drosophila* requires the formation of a precise pattern of differentiated cell types. The formation of this pattern begins in the eye imaginal disk of third instar larvae, where cells are progressively recruited from an unpatterned epithelium to form the individual units, or ommatidia, of the eye. Examination of individual cells in the forming ommatidia has shown that the photoreceptors differentiate in a fixed sequence, beginning with the central R8 photoreceptor and proceeding pairwise with R2 and R5, R3 and R4, R1 and R6, and finally R7. The recruitment and differentiation of these cells occurs in response to positional cues, generated, sensed, and responded to by cells in the developing field. Components of these signaling pathways can, in principle, be identified by isolating mutant flies in which the developmental process is interrupted at specific stages.

II. *Ellipse* and *scabrous* Genes: Spacing the R8 Cells.

An early step in the formation of ommatidia is the distribution of cells that will give rise to the R8 cells. Dr. Rubin's group is studying two mutations, *scabrous* and *Ellipse* (*Elp*), that affect this process in opposite ways. In *scabrous* mutants it appears that too many cells begin to differentiate as R8 cells, whereas in the mutant *Elp*, very few cells do. The *scabrous* gene has been cloned, and its structure and expression are being analyzed. From genetic studies, Dr. Rubin and his colleagues have demonstrated that *Elp* is a dominant mutation in the gene that encodes the *Drosophila* homologue of the mammalian epidermal growth factor receptor (DER). How ommatidia come to form in a regular spatial array is unknown, but local competition and lateral inhibition between disk cells may be involved. It is not known why *Elp* mutations predominantly affect the eye, when amorphic mutations are lethal to the embryo. Possibly the increased DER activity in *Elp* is specific to the developing eye, or the eye may be especially sensitive to such an increase. It is also not known whether *Elp* mutations increase the expression of DER protein or enhance its activity, perhaps with respect to an eye-specific ligand or target.

III. The *sevenless* Gene.

The best-characterized mutant affecting retinal development is *sevenless*. The *sevenless* gene is essential for the development of a single type of photoreceptor cell; in the absence of proper *sevenless* function the cells that would normally become the R7 photoreceptors become nonneuronal, lens-secreting cells. Previous morphological and genetic analyses have indicated that the product of the *sevenless* gene is involved in reading or interpreting the positional information that specifies this particular developmental pathway.

In previous reports Dr. Rubin's group described the isolation, nucleotide sequence, and expression pattern of the *sevenless* gene. The *sevenless* gene encodes a protein tyrosine kinase cell surface receptor that is expressed in a highly specific and complex pattern in the developing *Drosophila* eye. Changes in expression of the *sevenless* protein occur very rapidly; substantial differences in protein expression can be observed between neighboring ommatidia that are only ~2 h apart in the developmental sequence. The *sevenless* protein is the only example of a tyrosine kinase receptor in which such a dynamic pattern of expression in a developing tissue has been described. It was therefore of interest to establish the mechanism by which this cell-specific pattern is generated. To distinguish

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transcriptional control of protein distribution from other post-transcriptional mechanisms, the *sevenless* promoter was coupled to the reporter gene, *lacZ*, which encodes β-galactosidase. Expression of β-galactosidase was found to parallel the *sevenless* pattern described previously by Dr. Rubin's group, indicating that the cell-specific pattern of *sevenless* protein expression in wild-type eye disks is generated transcriptionally. This transcription pattern is controlled by an enhancer element located in an intron of the gene.

An understanding of the structure and activity of the *sevenless* protein is a prerequisite for understanding the molecular mechanisms of *sevenless*-mediated signaling. Dr. Rubin's group has expressed the *sevenless* protein in *Drosophila* tissue culture cells and studied its synthesis, processing, and activity. The *sevenless* protein possesses protein tyrosine kinase activity. The protein is first synthesized as a 280 kDa glycoprotein precursor that is subsequently cleaved into 220 kDa amino-terminal and 58 kDa carboxyl-terminal subunits that remain associated by noncovalent interactions. The 220 kDa subunit is glycosylated and contains most of the extracellular portion of the protein, whereas the 58 kDa subunit is composed of a small portion of the extracellular sequences and the intracellular protein tyrosine kinase domain. This complex is subsequently cleaved into either 49 or 48 kDa carboxyl-terminal fragments, with concomitant degradation of the rest of the protein.

The adoption of distinct fates by cells in the developing retina that are in such close proximity to one another must require precise cell-cell signaling mechanisms. This precision must result from the specific coincidence in time and space of ligands, receptors, and intercellular signal-transducing factors. In theory the required specificity could be achieved by regulation of any combination of these proteins. Dr. Rubin's group has begun to address this issue by replacing the restricted pattern of *sevenless* expression with one where the protein is expressed in all cells of the eye disk under the control of a heat-shock promoter. Consistent with a role in receiving a transient inductive signal, expression of *sevenless* was found to be required only during a brief period of ommatidial development for formation of the R7 cell. However, the complex spatial distribution of *sevenless* protein within the developing ommatidium is not a crucial part of the positional information that provides specificity to the signal mediated by *sevenless*, suggesting that the necessary specificity is provided by the temporal or spatial distribution of its ligand or substrate.

IV. Additional Genes Involved in Retinal Development.

Dr. Rubin and his colleagues are using both conventional mutagenesis and the P element–mediated enhancer detector method to carry out extensive genetic screens for mutations that affect development of the various cell types in the eye. They have isolated and are genetically and phenotypically characterizing several new mutations that affect ommatidial development. In addition, they are continuing their studies of the *rough* gene.

Dr. Rubin is also John D. MacArthur Professor of Genetics at the University of California at Berkeley and Adjunct Professor in the Department of Biochemistry and Biophysics at the University of California School of Medicine at San Francisco.

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SECOND MESSENGER MECHANISMS IN *APLYSIA* NEURONS

JAMES H. SCHWARTZ, M.D., PH.D., *Investigator*

Central to Dr. Schwartz's work is the identification of molecular events that control synaptic transmission. Signal transduction mechanisms are fundamental to understanding synaptic plasticity (the change of a neuron's activities as a result of prior stimulation or training), which is thought to underlie learning and memory. The principal second messenger systems that have been studied in identified *Aplysia* neurons are the cAMP cascade, the activation of protein kinase C, and the newly discovered action of lipoxygenase metabolites or arachidonic acid. Identification of a modulatory cascade and specification of its target substrates are not, however, sufficient for explaining memory and learning. Dr. Schwartz and his co-workers have concentrated on understanding the mechanisms by which the modulatory molecular events are made to endure in the neuron and thus how they might operate to change the behavior of the animal.

I. Cyclic AMP

One molecular mechanism for persistent protein phosphorylation had previously been demonstrated in Dr. Schwartz's laboratory: as a consequence of long-term training, regulatory (R) subunits of the cAMP-dependent protein kinase diminish in sensory neurons, whereas catalytic (C) subunits remain constant. To determine how this change in ratio of R-to-C subunits is produced, antibodies to both subunits are being raised (using sequence information obtained from last year's work on the cloning of these molecules), a synaptosome preparation from *Aplysia* ganglia has been developed, and it has been shown that synthesis of new protein is required for maintenance of the change in subunit ratios that occurs with long-term training.

The turnover of R does not occur after short-term training. This change in the R-to-C ratio provides a mechanism for the persistent phosphorylation observed by Dr. Eric R. Kandel (HHMI, Columbia University) in long-term, trained sensory neurons in which the level of cAMP is baseline, since decreasing the amount of R relative to C should increase kinase activity at any subsaturating concentration of cAMP (left shift). Dr. Kandel and his co-workers reported, however, that inhibitors of protein synthesis prevent the development of long-term sensitization and block persistent protein phosphorylation. In collaboration with Dr. Kandel, Dr. Schwartz and his colleagues examined whether the *lasting* disappearance of R subunits depends on new protein synthesis, with isolated sensory neuron clusters exposed to serotonin for 2 h, a protocol that produces facilitation lasting 24 h. This treatment reproduces the diminution of R subunits in sensory neuron clusters found in the intact animal when the sensory cells are tested 24 h after the exposure. (The isolated nervous tissue provides a more convenient experimental preparation to examine this phenomenon than does the intact animal.) If the diminution of R subunits is involved in producing long-term sensitization, their enduring loss would be expected to be blocked by the same inhibitors. Inclusion of anisomycin, an inhibitor of protein synthesis, during the exposure to serotonin blocked this effect, indicating that new protein must be made to maintain the diminished ratio of R-to-C subunits.

Although new protein synthesis is required for the sustained decrease in R-to-C ratio, the biochemical mechanism by which R subunits are made to diminish is post-translational, because it occurs in *Aplysia* synaptosomes (which do not make new protein) during continuous exposure to a permeating and effective analogue of cAMP, with no degradation of the C subunit or any other major protein. Dr. Schwartz now will determine the mechanisms by which the R-to-C ratio is altered and how that alteration is maintained. Preliminary experiments indicate that there is no change in messenger RNA for R subunits after long-term treatments, and this suggests that the alteration may be maintained by a change in RNA processing or the induction of a specific protease.

II. Protein Kinase C.

Behavioral training of the animal, as well as application of serotonin, produces the same translocation of protein kinase C from cytosol to membrane seen with phorbol esters, which Dr. Kandel and his co-workers have found to produce short-term facilitation. This translocation, which occurs directly in sensory cells, has been confirmed by measuring the kinase both by its enzymatic activity and by phorbol ester binding. Kinase activity on the membrane is constitutively active; that is, it no longer requires exogenous lipid activators. Dr.

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Schwartz and his co-workers have found that *Aplysia* contains at least two protein kinase C genes with four transcripts.

Like the predominant γ form in vertebrate nervous tissue, one *Aplysia* form can make use of arachidonic acid at low concentrations in place of diacylglycerol. The forms can be separated biochemically (differential association to membrane fractions in the presence of 10 mM Mg^{2+}), and by antibodies raised against peptides synthesized according to appropriate carboxyl-terminal amino acid sequences inferred from the cloning data.

Membranes of *Aplysia* neurons contain a M_r 30,000 protein that stimulates the activity of phospholipase A_2 (PLA_2) only when phosphorylated. This protein, which Dr. Schwartz and his colleagues have characterized and purified to homogeneity, using a two-step purification procedure [DEAE (dimethylaminoethyl) chromatography followed by size-exclusion HPLC (high-performance liquid chromatography)], is associated with *Aplysia* neural membranes. The purified protein can be phosphorylated *in vitro* by purified protein kinase C. Since sensitizing stimuli to the intact animal and application of serotonin to isolated clusters of sensory neurons activate protein kinase C by translocating the enzyme to membrane and since the kinase can cause the phosphorylation of the PLA_2-stimulating protein, it is attractive to think that the key biochemical event sustaining the facilitation is the initiation of a cycle that results in a *persistently* active protein kinase C, with arachidonic acid causing continued activation of the kinase at the presynaptic site in sensory neuron terminals to which it initially was translocated.

Dr. Schwartz is also Professor of Physiology and Cellular Biophysics and of Neurology at the Columbia University College of Physicians and Surgeons and Member of the Center for Neurobiology and Behavior, located at the New York Psychiatric Institute.

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MODULATION OF NEURONAL ACTIVITY BY INTRACELLULAR MESSENGERS

STEVEN A. SIEGELBAUM, PH.D., *Associate Investigator*

Dr. Siegelbaum has continued to focus on the molecular mechanism of ion channel modulation by second messengers in molluscan and vertebrate neurons. In addition, the role that intracellular calcium plays in both the induction and expression of neuromodulation has been studied.

Arachidonic acid and its lipoxygenase metabolites have recently been shown to serve as second messengers in neurons. Previously this laboratory, in collaboration with Drs. Eric R. Kandel and James H. Schwartz (HHMI, Columbia University), showed that the 12-lipoxygenase metabolite of arachidonic acid, 12-HPETE (12-hydroperoxyeicosatetraenoic acid), mediated an increase in opening of the S-type K^+ channel of *Aplysia* sensory neurons in response to the neuropeptide FMRFamide (Phe-Met-Arg-Phe-amide). The increase in K^+ current hyperpolarizes the neuron and contributes to presynaptic inhibition. During the past year this work has been extended by 1) investigating the mechanism whereby 12-HPETE causes S channel opening and 2) exploring the potential role of arachidonic acid metabolites as modulators of neuronal function in chick sympathetic neurons.

I. Modulation of S K^+ Channel Activity by 12-HPETE.

Four possible mechanisms by which 12-HPETE could modulate S channel activity include 1) protein kinase activation, leading to S channel phosphorylation; 2) phosphatase activation, leading to S channel dephosphorylation; 3) G protein activation, leading to direct G protein gating of the S channel; and 4) a direct action of 12-HPETE on S channel activation. To investigate these possibilities, Ned Buttner, Dr. Andrea Volterra, and Dr. Siegelbaum applied various arachidonic acid metabolites directly to cell-free patches of membrane that lack ATP or GTP. The results of these experiments show that 12-HPETE causes a dramatic and reversible increase in S channel opening in both cell-free inside-out and outside-out patches, where the inside or outside of the membrane faces the bathing solution. Moreover, this effect is specific for this particular metabolite, since 12-HETE (a stable breakdown product of 12-HPETE), arachidonic acid, or 15-HPETE have no effect on S channel activity. Since the bath contained neither ATP nor GTP, the effect of 12-HPETE cannot involve phosphorylation or G protein activation, and since the effect is readily reversible, phosphatase activation is also ruled out. Therefore it was concluded that 12-HPETE (or possibly some downstream metabolite) modulates S channel activity by acting directly on the S channel or a protein closely linked to the S channel in the cell-free patches. Surprisingly, 12-HPETE was effective at 100-fold lower concentrations when applied to outside-out patches compared with its action on inside-out patches. This suggests that the 12-HPETE receptor may be localized to the outer surface of the S channel.

II. Modulation of Calcium Channel Activity in Chick Sympathetic Neurons by Arachidonic Acid.

Do the arachidonic acid metabolites also modulate channel activity in vertebrate neurons? To address this question, Dr. Siegelbaum is studying the effects of arachidonic acid on calcium channel function in chick sympathetic neurons, in collaboration with Dr. Lorna Role (Columbia University), Dr. Linda Simmons, and Bill Bug. Many transmitters and peptides have been shown to produce presynaptic inhibition in sensory neurons through calcium current inhibition; however, the mechanism of this inhibitory action is unknown. The experiments of these investigators s how that arachidonic acid (0.3–10 μM) exerts a powerful inhibitory effect on the calcium current. A related fatty acid with but a single double bond (eicosamonoenoic acid) has no effect on the calcium current. Preliminary characterization of the calcium current suggests that it corresponds to a high-voltage–activated, dihydropyridine-insensitive type of calcium current.

III. Intracellular Calcium Modulation by Serotonin and FMRFamide.

The third project concerns the control and modulation of intracellular calcium concentration by neurotransmitters in *Aplysia* sensory neurons. Previous studies have shown that serotonin (5-HT) produces presynaptic facilitation, and FMRFamide produces presynaptic inhibition. It has been proposed that these actions are due to a modulation in the size of the internal calcium transient in response to action potentials. Using the fluorescent Ca^{2+} indicator Fura-2 and digital video imaging, Hal Blumenfeld and Dr. Siegelbaum have investi-

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gated this problem. Imaging of neuronal cell bodies, growth cones, or neurites of sensory neurons in culture consistently shows an increase in the size of the intracellular calcium transient in response to 5-HT and a decrease in the calcium transient in response to FMRFamide. The Ca^{2+} transient in response to action potentials is not uniform throughout the cell; there appear to be local hot spots of calcium. Moreover, the increase in the calcium transient with 5-HT is also not uniform. Some areas of the cell show significantly larger increases in calcium than others. What is most surprising is that the hot spots of Ca^{2+} do not necessarily coincide with the regions that show the largest response to 5-HT.

The modulation of the calcium transients by 5-HT and FMRFamide could be due to a modulation of calcium influx and/or calcium buffering. To assess the latter possibility, the effects of 5-HT were studied on the calcium transient produced in response to intracellular injection of inositol trisphosphate (IP_3), a second messenger that releases calcium from intracellular stores. IP_3 causes a significant calcium transient in the sensory neurons that is independent of external calcium. 5-HT has no effect on the IP_3-induced calcium transient, in contrast to its consistent potentiation of the action potential–induced calcium transient, which does depend on external calcium. Therefore the increase in the calcium transient with 5-HT is probably due to a modulation of calcium influx. Whether this results from a direct modulation of calcium channels or is an indirect effect of action potential broadening due to S K^+ channel closure by 5-HT is under study.

Dr. Siegelbaum is also Associate Professor of Pharmacology in the Center for Neurobiology and Behavior at the Columbia University College of Physicians and Surgeons.

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THE BIOLOGY OF SYNAPSES

STEPHEN J. SMITH, PH.D., *Associate Investigator*

Research in Dr. Smith's laboratory addresses the biology of synapses, with a special focus on the development of synaptic structure. Closely related efforts address mechanisms of intracellular signaling by the calcium ion, a cytoplasmic messenger that plays a major role in synaptic development and function. These studies employ cultured mammalian neurons and glial cells, slices and other forms of explanted mammalian brain tissue, and marine specimens, including the squid. The central methods include digital video light microscopy, confocal scanning laser microscopy, electron microscopy, immunohistochemistry, optical measurement of intracellular calcium, and electrophysiology.

I. Mechanisms of Growth Cone Motility.

The growth of nerve fibers is guided by a highly motile terminal specialization called the growth cone: its motility involves alternating protrusion and retraction phases that apparently explore and test embryonic surfaces. Recent experiments by Dr. Paul Forscher in this laboratory have employed digital video microscopy to discriminate between two general motility models that might apply to growth cones: one model is based on membrane traffic and the other on dynamics of the actin cytoskeleton. Dr. Forscher's results have strongly favored actin-based models and have led to a detailed model for growth cone motility that involves a combination of two actin-based mechanochemical mechanisms. In this model the protrusive phase of crawling is related primarily to the actin polymerization cycle, while the retraction phase reflects the actin-myosin interaction.

II. Neuronal Migration in Developing Telencephalon.

The neurons that populate layers of mature neocortex arise from mitoses of stem cells lining the cerebral ventricle and then migrate in a precisely choreographed fashion toward the pial surface. Cells destined for the most superficial layers arise later on and bypass older neurons that remain in deeper layers. Experiments carried out in this laboratory by Drs. Monica Cooper and Aaron Waxman may have taken a large step toward elucidating the mysterious basis for this migratory choreography. Employing the new techniques of laser confocal microscopy, they have developed the first means for live observation of cell migration within intact cortical tissue explants. These methods have yielded dynamic observations of subcellular details (e.g., growth cones, submicron neurites, single organelles, and individual filopodia) at depths up to 100 μm within acutely isolated brain tissue. They have also recorded intracellular calcium from individual neurons under similar circumstances.

III. Cell Motility and Long-Term Synaptic Plasticity.

Synapses in the mammalian hippocampus show a long-lasting form of functional plasticity called long-term potentiation (LTP). One hypothesis about LTP envisions a motility event somehow consolidated into a lasting structural change as the fundamental synaptic modification. Although there have been a few observations in support of this hypothesis, it has proved difficult to test decisively, because it has not been possible to visualize living synapses during LTP. Ongoing studies in this laboratory demonstrate that the new laser scanning confocal microscope may be capable of providing such a definitive test. With vital fluorescent staining, individual synaptic varicosities and dendritic spines have been resolved in good detail. Dr. Robert Goldman and Alex Chernjavsky have recently succeeded in marrying this new instrument to the type of electrical recording apparatus needed to induce and test LTP.

IV. Early Stages in Synapse Formation.

When an axonal growth cone contacts an appropriate target cell, the growth cone begins a transformation into the secretory presynaptic terminal, while the adjacent site on the target cell acquires the specialized features of the postsynaptic complex, including neurotransmitter receptors. Dr. Cooper has been working to elucidate details of this process as it occurs in cell culture. Her ongoing studies have revealed a striking and unexpected feature of the synaptogenic contact: the growth cone and target cell almost always make their initial contact at a site where the target cell dendrite is actively growing fine processes of some type. Thus the initial contact between an axonal growth cone and a target cell dendrite generally occurs at the tip of some fine branch of the dendrite, not on the

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dendritic shaft itself. After initial contact, however, the growth cone may migrate to a final position on the dendritic shaft or cell body before stabilizing and continuing to later stages of synapse formation.

Drs. Waxman and Cooper are also studying intracellular calcium transients associated with early stages of synapse formation in cell culture. They are using time-lapse video recording to image cultured hippocampal neurons loaded with the fluorescent calcium indicator Fluo-3. They have observed three different types of calcium signal at around the time of initial intercellular contact: 1) spontaneous calcium oscillations occurring prior to any distinguishable cell contact, 2) calcium elevations occurring at the very moment of initial contact, and 3) synchronized calcium oscillations in groups of cells that have already established contact. They are now investigating the mechanisms of these calcium signals and their significance in the processes of partner selection and synapse formation.

V. Localization of Calcium Channels at the Presynaptic Active Zone.

In studies of the squid giant synapse over the last three years, Dr. Smith and JoAnn Buchanan have been collaborating with Drs. George Augustine and Milton Charlton (Woods Hole) to study the molecular organization of the active zone at the squid giant synapse. The main thrust of this work has been to test earlier indications that the presynaptic calcium channels are highly localized to the active zone region. Two studies completed this year provide strong support for this channel localization hypothesis. A study correlating fluorescent measurements of the presynaptic calcium transient with detailed anatomical reconstructions found an excellent agreement between loci of calcium influx and ultrastructurally identified active zones; high-resolution observations with a laser confocal microscope have confirmed and extended earlier results from conventional fluorescence microscopy.

VI. Propagation of Calcium Signals Across Intercellular Gap Junctions.

Studies completed this year on two different systems have led to similar observations: intercellular propagation of a physiological calcium signal through a gap junction. In the first study, a collaboration with Drs. Paul Brehm, James Lechleiter, and Kathleen Dunlap (Woods Hole), digital imaging of endogenous, calcium-dependent bioluminescence in the hydrozoan coelenterate *Obelia* provided the evidence for intercellular propagation of voltage-induced calcium signals. The second study, conducted in Dr. Smith's laboratory by Steven Finkbeiner and Drs. Ann Cornell-Bell and Mark Cooper, grew from a discovery of glutamate-receptor–activated calcium signals in cultured hippocampal astrocytes. This team found that, under many conditions, glutamate-induced calcium signals propagated from cell to cell in confluent cultures and that this propagation occurs through gap junctions. For both *Obelia* and the astrocyte cultures, major questions remain as to whether the gap junctional signal is calcium itself or some other intermediate chemical message, such as inositol trisphosphate (IP_3).

Dr. Smith is also Associate Professor in the Section of Molecular Neurobiology at the Yale University School of Medicine.

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PROBABILISTIC FUNCTION OF CENTRAL SYNAPSES

CHARLES F. STEVENS, M.D., PH.D., *Investigator*

One fundamental characteristic of synaptic transmission revealed by studies of the neuromuscular junction is that neurotransmitter release is quantal and probabilistic: that is, the presynaptic terminal can release only integral multiples of a minimum quantity of transmitter, and the exact number of transmitter packets released varies in a random manner. This quantal release is viewed as reflecting the stochastically occurring exocitosis of neurotransmitter-containing vesicles. The ability to identify the probability of quantal release and the size of the individual quantum is crucial for the analysis of synaptic transmission. For example, when a synapse becomes stronger with use, is it the quantal size or the release probability (or both) that changes? The mechanism of synaptic strengthening cannot be elucidated without answering basic questions of this sort.

Although synaptic transmission in the central nervous system has also been reported to be quantal, no satisfactory analysis of probabilistic release at a central synapse has been accomplished. The difficulty has been technical. As part of their program to understand the mechanisms underlying neuronal information processing, Dr. Stevens and his colleagues have developed methods for carrying out quantal analysis of synaptic transmission between hippocampal neurons. The results have confirmed that the same formalism originally developed to describe release at the neuromuscular junction is adequate as well for these central synapses. A simplifying assumption made by all previous investigators—that the quantal size at an individual synapse is constant—has been shown, however, to be invalid. Thus a successful application of the original formalism for quantal analysis is more complicated for these interneuronal synapses than it is for the neuromuscular junction.

Two main problems have plagued quantal analysis in the central nervous system. First, synapses are widely distributed over the neuron's synaptic tree. Because of cable attenuation of events that occur at more remote dendritic locations, one cannot distinguish between fluctuations in the size of synaptic currents due to the site of synaptic contact from those that are reflections of the probabilistic nature of quantal release.

Second, the neuronal interconnections are complex. For a valid quantal analysis, only a single presynaptic neuron can be stimulated, so that the studied connection is restricted to just one synapse; but in the central nervous system, this is extremely difficult. To activate only a single cell, intracellular stimulation is required, but a given pair of neurons are connected so infrequently that the chances of recording simultaneously from an appropriate pair are remote.

The first requirement of quantal analysis is that individual quanta, miniature synaptic currents (minis), be identified. Isolated minis are seen in central neurons as spontaneously occurring small transient currents that continue to appear when evoked synaptic transmission is blocked (with tetrodotoxin, low calcium/high magnesium, or presence of cadmium ions, for example). These minis are variable in size and shape, however, and one cannot generally know what fraction of the variability is inherent and what fraction is the result of cable attenuation of events that occur far out the dendritic tree.

Dr. Stevens and his colleagues have circumvented these problems by using cultures of rat hippocampal neurons and evoking mini release with locally applied hypertonic solution. By correlating the efficacy with which hypertonic solution produces minis with the presence of boutons (revealed by synapsin immunohistochemical localization), the laboratory was able to show that the hypertonic solution action is restricted to the immediate site of application. The fact that minis could be evoked at any specified location on the dendritic tree made two important classes of observations possible. 1) The laboratory was able to determine the size distribution of minis that occurred adjacent to the recording site so that sizes were unaffected by cable filtering. 2) Precisely what effect cable filtering had on the size and shape of minis could be determined by causing them to occur at different but known locations on the dendritic tree. The cable theory could be used to describe these effects quantitatively. Because the shape of minis is dramatically altered by cable filtering, the site of origin of a mini can be identified from its shape.

Evoked release is conveniently studied in culture, because a high fraction of the adjacent neurons are connected. Furthermore, synaptic contacts that one cell makes on another usually are all formed at one site of the dendritic tree, and if multiple contacts are made, this can be detected by direct visualization (by dye-filling both neurons that are being

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studied) and from the shape of the minis. The distance out the dendritic tree of the synapse that produces each mini is signaled by the mini shape, so synaptic contacts separated on dendrites are easily detected. The laboratory, then, has been able to carry out quantal analysis of synaptic transmission between hippocampal neurons in culture.

Although minis at the neuromuscular junction vary little in size, the quantal size observed in hippocampal neuronal connections varies considerably, and one quantum is frequently two or three times as large as another. The shape of the mini size distribution is not Gaussian, but rather is skewed with a long tail to the larger minis. This distribution can be accounted for quantitatively by the distribution of vesicle sizes observed in electron micrographs of cultured neurons. Because the minis vary so much in size, the equations that are used to determine quantal release parameters (probability of release and number of quanta ready for release) must be modified.

Evoked release shows conspicuous fluctuations in the amplitude of individual currents, including occasional failures of release. These fluctuations have been studied for various extracellular magnesium concentrations (a standard way to modify release probability); a standard theory assuming that the number of quanta released follows a binomial distribution provides an accurate description of the data, once the formalism is modified to take account of the large fluctuations in mini amplitude. The agreement between observed and predicted synaptic current magnitudes is excellent.

These experiments are the first satisfactory quantal analysis of central synaptic transmission. An important aspect of this work is the finding that central quanta vary considerably in size, at least in culture, so that this must be taken into account in the application of the theory. One important issue in long-term potentiation is whether the mechanism responsible for increased synaptic strength is pre- or postsynaptic. The only definitive way to determine the extent to which an increased efficacy in synaptic transmission is due to increased response to quanta or to altered release probability (to mention only two of the obvious possibilities) is through quantal analysis. Dr. Stevens and his colleagues plan to use their modified equations describing synaptic release to investigate this question.

Dr. Stevens is also Professor of Molecular Neurobiology at Yale University School of Medicine.

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BODY PATTERNING IN *DROSOPHILA* EMBRYOS

GARY STRUHL, PH.D., *Assistant Investigator*

Dr. Struhl's research is focused on the molecular nature and mode of action of spatial determinants responsible for segregating the early *Drosophila* embryo into its characteristic pattern of body segments. Both classic embryological and genetic experiments have shown that the segment pattern is prefigured at fertilization by distinct anterior, posterior, and terminal determinant systems laid down in the egg during oogenesis. The roles of each of these systems, as well as the molecular mechanisms involved in their initial establishment and subsequent function, have been investigated.

I. Anterior Determinant System.

Prior work by Dr. Christiane Nüsslein-Volhard and her colleagues has established that a single morphogen, *bicoid* (*bcd*), controls anterior body pattern. Transcripts of the gene are synthesized in the nurse cells and then transported to the oocyte, where they appear to be trapped at their point of entry—the future anterior end of the embryo. After fertilization these tightly localized transcripts are translated, and the resulting protein diffuses from its site of synthesis, generating a concentration gradient. The *bcd* gradient then dictates anterior body pattern, by controlling where subordinate regulatory molecules are expressed.

Recent studies in this laboratory have shown that the 3′-noncoding portion of the *bcd* transcript contains a cis-acting signal that is both necessary and sufficient to allow mRNAs synthesized in the nurse cells to be trapped selectively upon entry at the anterior pole of the oocyte. This large (625 bp) localization signal is likely to form an extensive secondary structure, suggesting that it acts as a ligand that is bound and anchored by a receptor protein in the oocyte. Thus establishment of the *bcd* morphogen gradient may depend on the selective retention of *bcd* transcripts by specialized receptor proteins as the transcripts pass from the nurse cells into the oocyte.

The *bcd* protein contains a homeobox domain, a structural motif associated with site-specific DNA binding in prokaryotes, yeast, and higher eukaryotes, suggesting that it might function as a transcription factor. Hence the graded distribution of *bcd* protein might determine where subordinate regulatory genes are expressed, by virtue of its ability to activate or repress transcription directly in a concentration-dependent fashion. Dr. Herbert Jäckle and his colleagues have recently shown that the regulatory gene *hunchback* (*hb*) is normally activated in a *bcd*-dependent fashion in a broad but sharply bounded anterior domain in early embryos, suggesting that it may respond to the *bcd* gradient by this mechanism.

The possibility that the *bcd* morphogen acts as a concentration-dependent transcriptional regulator has been tested directly by creating a series of hybrid genes in which small portions of the upstream regulatory region of *hb* have been inserted in front of a naive transcriptional start site, driving expression of the coding sequence for β-galactosidase. When returned to the genome, a subset of these hybrid genes were expressed in sharply bounded anterior domains specified by the *bcd* gradient (like the endogeneous *hb* gene). All of the hybrid genes in this subset contained specific cis-acting elements found within a small portion (250 bp) of the *hb* upstream regulatory region. Furthermore, it was possible to reconstruct *bcd*-dependent transcriptional activation mediated by these same elements in yeast, a heterologous system lacking all other *Drosophila* components. These studies provide a strong argument that direct interactions between *bcd* protein and discrete cis-acting regulatory elements in the *hb* gene are responsible for controlling where the *hb* gene is normally expressed during early embryogenesis. It was also shown that the posterior boundaries of expression of the various hybrid genes were determined by the number and quality of the cis-acting elements mediating their response to *bcd* protein. This result provides direct evidence that the *bcd* gradient can dictate several distinct domains of anterior gene expression, depending on the number or quality of the regulatory sites governing the response of particular target genes. Preliminary genetic and molecular studies have suggested several other candidate genes that normally may respond directly to the *bcd* gradient.

II. Posterior Determinant System.

Control of posterior body pattern was initially thought to occur in a symmetrical fashion to that of anterior body pattern, depending on an opposing gradient of the morphogen *nanos*. However, in work begun during the previous year, a surprising result was obtained that suggested that this view is

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incorrect. The spatial signaling roles of zygotic control genes such as *hb* were examined, by forcing them to be expressed in portions of the embryo where they are normally inactive. In the case of *hb*, this was achieved by placing the *hb* gene under the control of the *hsp70* promoter and heat-shocking early embryos to drive ubiquitous *hb* protein expression. Surprisingly, ubiquitous *hb* expression caused a phenotype that appears indistinguishable from that caused by mutations that block activity of the *nanos* morphogen. This unexpected result focused attention on the prior demonstration by Dr. Diethard Tautz that the *nanos* morphogen normally blocks expression of *hb* protein in the posterior half of the body by destabilizing maternal transcripts of the gene that are initially distributed throughout the fertilized egg. These findings suggested that the *nanos* morphogen may normally have only a single role-blocking posterior expression of *hb* protein from maternally derived transcripts. This possibility has been shown to be correct by eliminating the maternally derived activities of both the *hb* and *nanos* gene products. Embryos that lack both activities give rise to normal larvae and adults.

The demonstration that early embryos lacking the primary posterior determinant system can develop normal body patterns under some conditions leads to the surprising conclusion that the *nanos* morphogen system is not directly responsible for organizing posterior body pattern. Instead, it seems to play a permissive rather than an instructive role. This is in striking contrast to the *bcd* morphogen system, which dictates anterior body pattern via its multiple direct effects on subordinate signaling molecules (e.g., transcription activation of the *hb* gene) and poses the question of what factors are responsible for specifying the posterior pattern. Preliminary experiments in which the *hsp70* promoter is used to drive ubiquitous expression of other early control genes, such as *Krüppel* and *knirps*, suggest that the local distributions of their protein products specify posterior body pattern. The mechanisms responsible for ensuring the orderly expression of these gene products in the absence of any single morphogen gradient comparable to the anterior *bcd* gradient are under investigation.

III. Terminal System.

Recent studies of Drs. Trudi Schüpbach, Eric Wieshaus, Christiane Nüsslein-Volhard, and their colleagues have established the existence of a third primary determinant system that distinguishes end portions of the body pattern from the middle portion. Moreover, the primary morphogen in this system appears to be encoded by the *torso* gene, which encodes a receptor tyrosine kinase.

In studies performed in this laboratory, the *torso* protein has been shown to be expressed ubiquitously on the surface of early embryos, despite genetic experiments that indicate that it normally acts selectively at both poles but must be inactive in the middle portion of the body. This finding argues strongly that the *torso* protein functions as a ubiquitous surface receptor that is activated only in the vicinity of the poles. Additional genetic and immunohistochemical experiments have confirmed this conclusion and suggested that the *torso* protein is activated by a localized ligand tethered to the extracellular matrix surrounding the early embryo. Finally, a mutation conferring temperature-sensitive, ligand-independent activity of the *torso* protein has been used to show that different levels of activity of the protein can specify the development of different portions of the terminal body pattern—progressively higher levels specify successively more terminal structures. This suggests that localized activity of the *torso* kinase triggered at both ends of the body is translated into opposing gradients of intracellular signaling molecules—possibly phosphorylated substrates of the kinase—which govern the specification of the anterior and posterior terminalia.

Dr. Struhl is also Assistant Professor of Genetics and Development at the Columbia University College of Physicians and Surgeons.

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SECRETORY PATHWAYS IN NEURONS

THOMAS C. SÜDHOF, M.D., *Associate Investigator*

Information is transferred between neurons at the synapse. Here neurotransmitters are released by the presynaptic cell and upon binding to receptors evoke a variety of postsynaptic responses. The presynaptic nerve terminal is a highly complex cellular structure. It synthesizes neurotransmitters and packages them into synaptic vesicles. These are targeted to the release site, secrete the neurotransmitters by exocytosis in a tightly regulated manner, and recycle. An understanding of the biological mechanisms by which neurotransmission occurs is dependent on understanding what happens during neurotransmitter release. The study of the release of neurotransmitters operates at the interface between cell biology and neurobiology; its results will be important for concepts about protein sorting and organelle biogenesis in eukaryotic cells and for elucidating synaptogenesis and the regulation of neurotransmission in the nervous system.

Dr. Südhof and his colleagues have embarked on a program to study the synaptic vesicle as the central organelle in presynaptic function. The first aim is to define the proteins of synaptic vesicles, their structures and localization. This has been achieved to the extent that several of these proteins have been purified, cloned, and expressed. The next aim is to determine how these proteins participate in the function of synaptic vesicles. Initial studies have provided insight into how synaptic vesicles may be formed and how their proteins may interact at the synaptic vesicle surface.

The predominant type of synaptic vesicle in vertebrate brain is the small translucent synaptic vesicle. These vesicles accumulate at the presynaptic density and primarily contain amino acid neurotransmitters, but not neuropeptides. The membranes of the small translucent synaptic vesicles exhibit a characteristic pattern of proteins. There are four major extrinsic membrane proteins that are phosphorylated upon neuronal stimulation. These extrinsic phosphoproteins, which are closely related to each other, are synapsins Ia, Ib, IIa, and IIb. Previous work has established that synapsins Ia and Ib bind to several elements of the cytoskeleton and that the four synapsins together account for ~10% of the total synaptic vesicle membrane protein. In collaboration with Dr. Paul Greengard, Dr. Südhof and his colleagues characterized the synapsins by molecular cloning and expression. The synapsins were found to be the differentially spliced products of two genes, one of which encodes synapsins Ia and Ib; the other encodes synapsins IIa and IIb. The amino-terminal regions of all four synapsins are highly homologous to each other and share a similar phosphorylation site for cAMP-dependent protein kinase and Ca^{2+} calmodulin-dependent protein kinase I. The carboxyl-terminal regions diverge in a manner such that each synapsin has a different set of shared or individual domains. The four synapsins were distributed differentially among synapses in rat brain. Their structures, distributions, and biochemical properties suggest a role in connecting synaptic vesicles to each other and to the cytoskeleton in a regulated manner. It is hypothesized that the amino-terminal domain shared by all synapsins represents the active site for these binding activities, which is differentially modulated in the four synapsins by the different carboxyl-terminal domains and by phosphorylation. Different synapses might have different characteristics dependent on the pattern of synapsins they express. Studies are under way to determine if changes in neurotransmitter release at a synapse are associated with changes in the expression of synapsins. The laboratory is also attempting to characterize the human genes for the four synapsins and to correlate their structures with the binding activities of the synapsins to synaptic vesicles and to the cytoskeleton.

Functionally, synaptic vesicles have an active proton pump of the endomembrane type and neurotransmitter uptake systems that depend on the transmembrane energy gradient established by the proton pump. The synaptic vesicle proton pump appears to be identical to that observed in clathrin-coated vesicles and chromaffin granules. The primary structure of one of its subunits from bovine brain was determined and found to be highly homologous to a subunit of the archibacterial proton ATPase, but not to the bacterial or mitochondrial F1,F0 proton ATPase, suggesting that the synaptic vesicle proton pump is similar to if not identical with a ubiquitous eukaryotic enzyme. This enzyme has evolved from a precursor shared with archibacteria but not with eubacteria. At this point the proton pump is the only identified synaptic vesicle protein that is not specific to synaptic vesicles in neurons but generally shared by intracellular organelles.

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Synaptic vesicles contain a characteristic pattern of intrinsic membrane proteins, of which proteins with apparent molecular weights of 65, 38 (synaptophysin), and 18 kDa (synaptobrevin) are major constituents and are being intensely studied in Dr. Südhof's laboratory. They have been purified, cloned, and expressed. Specific antibodies have been raised against each of them, and their membrane topology and possible functions are being investigated. All three proteins are comparatively abundant in synaptic vesicles but completely absent from other neuronal organelles. They are present in all synaptic vesicles independent of their neurotransmitter type and do not represent subunits of the proton pump or neurotransmitter transporters. Presumably these proteins have a function that is specific to synaptic vesicles but independent of neurotransmitter content, a function that may be related to the cell biological life cycle of the vesicles.

Synaptophysin is an evolutionarily well-conserved synaptic vesicle protein that contains four transmembrane regions and a tyrosine-rich repeated sequence at its carboxyl terminus that is probably phosphorylated. Specific antipeptide antibodies and limited proteolysis of synaptic vesicles were used to establish its transmembrane topology. Synaptophysin is known to form higher molecular weight complexes, and it has been hypothesized that it might form a transient pore in the membrane during exocytosis. The self-association of synaptophysin in the synaptic vesicle membrane is currently being studied to determine if a membranous pore is formed that is lined by transmembrane regions or if the self-association is mediated by the extramembranous parts of the protein to form a kind of membrane skeleton.

Synaptic vesicle membrane proteins are expressed not only in neurons but also in endocrine cells. Analysis of the expression of different synaptic vesicle proteins in endocrine cells revealed a ratio between the synaptic vesicle proteins that is different from that observed in native synaptic vesicles. While the expression of p65, synaptobrevin, and the four synapsins can be demonstrated in endocrine cells but appears to be low, synaptophysin is expressed at levels comparable with those observed in brain. Upon analysis by immunocytochemistry or subcellular subfractionation, >95% of the synaptic vesicle membrane protein in endocrine cells was localized to a small vesicular compartment distinct from the large secretory granules. The secretory granules in endocrine cells contained low-to-undetectable amounts. Surprisingly, when synaptophysin was expressed by transfection in fibroblastic CHO cells, it was also targeted into a small vesicular compartment indistinguishable from that observed in endocrine cells. In collaboration with Drs. Pietro DeCamilli and Reinhard Jahn, biochemical and immunocytochemical techniques were used to identify the synaptophysin-containing compartment in endocrine and fibroblastic cells. This compartment corresponds to the vesicular pathway of recycling receptors, such as the transferring receptor. The receptor-mediated endocytosis pathway shares with synaptic vesicles the property of recycling independently of the Golgi complex. The targeting of synaptic vesicle proteins into this pathway in endocrine cells suggests that the pathway of synaptic vesicles may represent a specialization of that of recycling receptors.

Synaptobrevin is an 18 kDa membrane protein of synaptic vesicles that has a single transmembrane region at its carboxyl terminus. A homologue of this protein was cloned from *Drosophila melanogaster* in Dr. Südhof's laboratory. Of the characterized synaptic vesicle proteins, synaptobrevin is the most conserved. It contains an evolutionarily poorly conserved amino terminus that is rich in proline or asparagine, followed by a highly conserved 70–amino acid sequence (78% identity between the *Drosophila* and bovine sequences). The structure of the protein suggests that the conserved region is located at the interface between the synaptic vesicle lipid bilayer and the cytoplasm, while the carboxyl-terminal halves of the transmembrane region and the small intravesicular sequences are not as well conserved. Although the function of synaptobrevin is unknown, its structure on the cytoplasmic face of the synaptic vesicle suggests that it serves to bind an unidentified cellular factor. Efforts are under way in Dr. Südhof's laboratory to use recombinant proteins to identify possible ligands for synaptobrevin.

The work in Dr. Südhof's laboratory has resulted in the characterization of several major components of synaptic vesicles and determination of the relation of the synaptic vesicle pathway to the general membrane traffic in eukaryotic cells. Future work will focus on understanding the interactions of synaptic vesicle proteins with each other and with other cellular proteins.

Dr. Südhof is also Associate Professor of Molecular Genetics at the University of Texas Southwestern Medical Center at Dallas.

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NEURAL CIRCUITS AND BEHAVIOR

LARRY W. SWANSON, PH.D., *Investigator*

The long-range goal of Dr. Swanson's laboratory is to clarify the functional organization of neural circuits that underlie changes in behavioral state. The approach has been first to define, with axonal transport methods, specific pathways in the rat central nervous system, and to examine the regulation of neurotransmission within specific components of this circuitry, using techniques for the cellular localization of transmitters, hormones, and receptors, as well as their respective mRNAs. Current studies are based on a comparison of circuits mediating three interrelated classes of behavior essential for survival of the individual and the species. These include drinking behavior (thirst) with associated cardiovascular regulation, eating behavior (hunger) with associated regulation of metabolism, and reproductive behavior and physiology. Two parts of the forebrain, the hypothalamus and limbic region, appear to play a fundamental role in mediating these behavioral and physiological responses, although unifying principles underlying their development and mature functional organization remain to be elaborated.

I. Reproductive Behavior.

Olfactory, and in particular pheromonal, information plays an important role in modulating reproductive physiology and behavior. Previous work in the laboratory showed that three cell groups, the posterodorsal medial nucleus of the amygdala, the encapsulated part of the bed nucleus of the stria terminalis, and the central part of the medial preoptic nucleus, appear to relay pheromonal information and contain many estrogen-concentrating neurons. Further work also showed that estrogen dramatically influences levels of the neuropeptide cholecystokinin (CCK) in all three cell groups in the female rat.

Double-immunostaining methods have now shown that many CCK-expressing neurons in these three cell groups also synthesize substance P (SP) and that estrogen influences CCK but not SP levels. Furthermore, *in situ* hybridization methods have been used to demonstrate that these effects of estrogen are accompanied by changes in levels of mRNA for CCK but not SP. Therefore estrogen appears to alter the ratios of CCK and SP mRNA and peptide within pheromonal sensory pathways to the hypothalamus. Since this effect was observed in intact cycling female animals, it appears to occur under physiological conditions. These results support the hypothesis that steroid hormones may effect a form of "biochemical switching" in anatomically fixed circuitry related to hypothalamic-mediated reproductive and ingestive behaviors.

A series of detailed cyto- and chemoarchitectonic studies was also carried out to subdivide the bed nuclei of the stria terminalis. This complex region adjacent to the hypothalamus is known from functional studies to play a key role in motivated behavior but has never been examined thoroughly. The results indicated that it can be divided into some 15 clearly distinct cell groups, whose connections must now be examined.

II. Neuronal Nicotinic Receptors.

Work during the last year has continued to characterize and localize neuronal nicotinic acetylcholine receptors (NAChRs) in the mammalian brain. It is clear from work at The Salk Institute that neuronal NAChRs are distinct from muscle and electric organ NAChRs. Neuronal receptors appear to consist only of α- (agonist-binding) and β- (non-agonist-binding) subunits, and these subunits are derived from different genes than muscle α- and β-subunits. In collaborative studies with Drs. Jim Patrick and Steve Heinemann and their colleagues, the neuronal β_2- and $\alpha_{2\text{-}4}$-subunits were characterized, and their distribution throughout the central nervous system was studied using *in situ* hybridization. The results indicate that the β_2-subunit is expressed in virtually every part of the brain and spinal cord, although the abundance varies widely in different cell groups. On the other hand, each α-subunit displays a unique, more restricted pattern of expression, although some areas clearly express more than one α-subunit. Additional subunits are being characterized, along with cloned subunits of the neuronal α-bungarotoxin–binding protein (in collaboration with Dr. Jon Lindstrom).

III. Transcription Factors.

A major goal of the laboratory is to clarify mechanisms underlying the influence of hormones on neuropeptide- and neurotransmitter-related receptors in circuits underlying motivated behavior. Dur-

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ing the past year, significant progress has been made in two relevant areas.

First, the distribution of the mineralocorticoid receptor throughout the brain has been determined using *in situ* hybridization, and the cis-trans-cotransfection assay has been used to show that mineralocorticoids and glucocorticoids may interact with the same promoter elements to expand the dynamic range of such elements to steroid hormones. These results may have important implications for understanding neural mechanisms underlying salt appetite, an essential component of ingestive behavior.

Second, in collaboration with Dr. Michael G. Rosenfeld (HHMI, University of California at San Diego), a family of transcription factors that may play an important role in the development of the nervous system has been identified. These nuclear proteins share a POU-domain, and some members also appear to be involved in establishing the phenotype of cells in other tissues as well, including the anterior pituitary, immune system, gonads, and kidney.

Dr. Swanson is also Senior Member at The Salk Institute for Biological Studies and Adjunct Professor of Neurosciences at the University of California at San Diego.

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VISUAL TRANSDUCTION IN RETINA

KING-WAI YAU, PH.D., *Investigator*

Dr. Yau's research is focused on the process of visual transduction in the retina. Vision begins in the retinal rod and cone receptors, where light is absorbed and transduced into an electrical signal. This signal, consisting of a graded membrane hyperpolarization, is then transmitted to second-order visual neurons by way of modulating the release of synaptic transmitter from the photoreceptor: in darkness the rate of transmitter release is high, and in the light this release is reduced in a graded fashion by the membrane hyperpolarization. Rods and cones, which subserve vision in dim and bright light, respectively, differ from each other: rods are ~100 times more sensitive to light than cones. The responses of rods to a flash of light also last longer, so that temporal integration is more effective, permitting rods to show an even higher sensitivity to steps of light.

In recent years much progress has been made in understanding the transduction process in rods. It is now known that the light-sensitive conductance, the closure of which in the light produces electrical hyperpolarization, is kept open in the dark by cyclic GMP (cGMP). Light, on the other hand, activates a specific phosphodiesterase to reduce the level of intracellular cGMP, thus leading to conductance closure. The activation of this phosphodiesterase involves photoisomerized rhodopsin and a GTP-binding protein. In cones the transduction process also seems to be similar qualitatively. Recently, Dr. Yau's laboratory has also shown that, in both rods and cones, the closure of the light-sensitive conductance triggers a negative-feedback regulation, mediated by a decrease in the intracellular concentration of free calcium ions, that rapidly reduces the gain of the phototransduction process. This feedback appears to play an important role in the well-known phenomenon of light adaptation, because when the feedback is experimentally removed, the photoreceptor cells lose most of their ability to adapt to background light.

During the past year the laboratory has been engaged in two investigations: 1) examination of the adaptation behavior of mammalian rod receptors to background light and 2) study of the gating characteristics of the cation channel that mediates the phototransduction process.

I. Background Adaptation in Mammalian Rods.

One important attribute of the visual system is the property of light adaptation, which allows the visual system to maintain the ability to detect contrast, despite large changes in the light level. In cold-blooded vertebrates, part of the ability of the visual system to adapt to background light resides in the rod receptors themselves. In mammals, on the other hand, the widespread belief is that the rod receptors scarcely adapt at all, leaving the task to postreceptor elements. Recently, Dr. Yau and his colleagues found that, in cold-blooded vertebrates, an important mechanism underlying receptor adaptation comes from a negative feedback mediated by intracellular calcium changes. Furthermore, this feedback serves an important function in darkness by stabilizing the steady ionic current through the cGMP-gated conductance and therefore maintaining the well-being of the cells. Thus the absence of background adaptation in mammalian rod receptors implies the lack of such a calcium feedback, which would jeopardize the ion homeostasis in these cells in darkness as well. This situation seems highly undesirable and unlikely. To settle this question, Dr. Yau and his colleagues undertook a comprehensive study of rod cells from a variety of mammalian species, including rabbit, cattle, cat, rat, and several primate species. In all cases the property of adaptation to background light was observed, much like in rods of lower vertebrates. Thus the previous belief of others is proven wrong.

II. Gating Characteristics of the cGMP-gated Channel.

The cGMP-activated conductance mediating phototransduction in retinal rods and cones represents a member (another member is the ion channel mediating olfactory transduction) of a novel class of ion channels that are gated directly by cyclic nucleotides acting as ligands. The details of the gating process, in particular the kinetics, however, remain unclear for any of these channels. Dr. Yau's group has undertaken a detailed study of the cGMP-gated conductance in cones by recording the openings and closings of single channels from excised patches of cone outer segment plasma membrane; all divalent cations were removed, because otherwise the channels would be blocked and the amplitude of the open-channel current reduced to a level below detection threshold. In addition to studying the channel kinetics at low cGMP concentrations, they have also examined the dependence of chan-

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nel activity on cGMP concentration all the way to saturation. From these experiments, they concluded that each channel molecule has perhaps five binding sites for cGMP. When fully liganded, the channel switches rapidly and repeatedly between the closed state and an open state of conductance near 50 pS. The opening and closing rate constants are of the order of 10^4 s^{-1} and 10^3 s^{-1}, respectively. In addition, at least one of the partially liganded states (most probably the next-to-full liganded state) likely can transit into a smaller open state of conductance 15–20 pS. Direct transitions between the large and the small open states may also occur, through gaining or losing of liganded cGMP. Computer simulation based on a simple kinetic scheme incorporating the above features broadly reproduces the temporal features exhibited by the recorded channel openings.

A prominent feature of the channel that comes out of this study is that the liganded channel opens and closes extremely rapidly (with both time constants <1 ms). Moreover, the liganded cGMP also unbinds from the channel very quickly (with a time constant also <1 ms). These properties enable the channel to act as a rapid sensor for the decrease in free cGMP in rods and cones during illumination. Finally, this channel, unlike many other ligand-gated channels, does not show desensitization to ligand. This unusual property, however, is important for phototransduction, by allowing a continuous dark current to be sustained by a steady basal level of free cGMP in the cells, and stopped only by light.

Dr. Yau is also Professor of Neuroscience at The Johns Hopkins University School of Medicine.

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MOLECULAR MECHANISMS OF ION CHANNEL FUNCTION

GARY YELLEN, PH.D., *Assistant Investigator*

Ion channels are integral membrane proteins that determine the electrical properties of neurons and other excitable cells. Chemically gated channels transduce neurotransmitter release at a synapse into an electrical signal; electrically gated channels modulate this electrical signal, propagate it over long distances, and convert this electrical signal (in the form of ion flux, particularly calcium influx) into a signal for neurotransmitter release at a second synapse or for cellular regulation. There are dozens of different types of ion channels, differing in their mode of regulation and in which ions they permit to pass.

Dr. Yellen's research is directed at the basic mechanisms of gating and permeation in ion channels. How are these proteins engineered to permit the controlled opening and closing of an aqueous pore that is capable of selecting between small ions of similar size, and what structural features of these proteins are responsible for the differences between different channels? Dr. Yellen's laboratory is using both molecular biological approaches and more traditional electrophysiological approaches to answer these questions.

I. Cloning of an Important Functional Variant of the Nicotinic Acetylcholine Receptor.

Natural evolution of ion channel proteins can give significant clues to the functional organization of these proteins. Nature has provided an important functional variant of the "normal" nicotinic acetylcholine receptor (AChR) found in vertebrate muscle and brain. The normal nicotinic AChR contains an ion channel that allows Na^+ and other cations to carry current through the membrane. In the sea slug *Aplysia* and other molluscan species, there is a similar nicotinic receptor that contains an anion-selective channel. This channel, like its cation-selective vertebrate counterpart, is inhibited by the snake toxin α-bungarotoxin.

By using a combination of homology-based cloning and protein chemistry, Dr. Yellen and Dr. James McLaughlin are cloning the cDNAs that code for this AChR variant in *Aplysia*.

II. Site-directed Mutagenesis of the Nicotinic AChR.

Site-directed mutagenesis is one important tool for establishing links between specific structural features of the channel protein and specific functions. Dr. Yellen, Mark Jurman, and Dr. Gordon F. Tomaselli have been applying this method to the *Torpedo* nicotinic AChR. Oligonucleotide-directed mutations are produced *in vitro*, and the mutant channels are expressed by injection of mRNA into *Xenopus* oocytes. Findings from other laboratories show that changes in charged residues just outside one of the transmembrane hydrophobic regions of the protein produce a change in single-channel conductance. Dr. Yellen is extending these findings by altering other charged residues and determining the changes in single-channel conductance at very low ionic strength. At low ionic strength, the sphere of influence of each charged group is dramatically increased. The effect of more distant charges can therefore be seen and their distances mapped by varying the ionic strength. This approach should give additional information about channel topology, since the effects of charge on the intracellular and extracellular sides of the channel can be distinguished.

Another set of site-directed mutations of the AChR is being prepared and tested in collaboration with Dr. Richard Huganir (HHMI, The Johns Hopkins University). Dr. Huganir has found that specific amino acids in the AChR can be phosphorylated by protein kinases and that this phosphorylation accelerates receptor desensitization. Although it is difficult to prepare completely unphosphorylated receptor from tissue sources, it is possible to produce mutant receptors that cannot be phosphorylated. The goal of this experiment is to determine the features of desensitization in completely unphosphorylated channels and then to see how the remaining desensitization is affected by charged amino acids (as opposed to phosphate groups). Mutations in several of the subunits have been prepared, and the mutant mRNAs are being tested in the oocyte expression system. It is now known that although phosphorylation can affect the rate of desensitization, it is not required for desensitization: desensitization still occurs when all of the potential phosphorylation sites are removed from the protein.

III. Selectable Expression Systems for Ion Channel Genes.

Although site-directed mutagenesis of large proteins can be used to advantage, it requires a specific testable model. Specific biophysical theories (e.g., surface charge effects on permeation) or protein

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chemical information (e.g., phosphorylation sites) allows the construction of such models, but complex phenomena such as ligand-dependent channel gating are probably more delocalized within the protein and are harder to guess at. Ideally, one could randomly mutagenize a large protein extensively, select mutants that exhibit a particular functional difference, and then determine the location and nature of the mutants. This procedure would give relatively unbiased and model-independent information about the parts of the protein important for this function.

To this end, Dr. Yellen's laboratory has been working on expression systems for ion channels that could be used for such a purpose. Previously, the yeast *Saccharomyces cerevisiae* was tried as a selectable expression system. It was possible to produce all four subunit proteins of the *Torpedo* nicotinic AChR in yeast cells, but no ion channel function was detected. For that reason, Dr. Yellen and Dr. Mark West have turned to mammalian cell expression systems, which have successfully expressed other similar ion channel proteins.

IV. Biophysical Studies on Reconstituted Channels.

Functional studies on single ion channels still provide the most detailed clues into the operation of channel proteins. Dr. Yellen and Susan Demo have been examining the interaction of permeant ions with the gating mechanism of the calcium-activated potassium channel from rat muscle, reconstituted in planar lipid bilayers. When the occupancy of the pore is increased by raising the concentration of potassium or rubidium, the channels tend to stay open more of the time. This observation is consistent with a "foot in the door" model that supposes that a channel cannot close when occupied by an ion. Other results with ammonium ions, however, suggest that occupancy of the channel cannot by itself explain the effect on gating and that the identity of the occupying ion and the particular location occupied within the channel are also important. Cesium ions, which block this channel, produce the same effect in a highly voltage-dependent manner. By correlating the voltage dependence of the gating effect with the voltage dependence of the block, Demo has shown that the gating perturbation is not a general allosteric effect but a specific effect of ions in the permeation pathway.

Dr. Yellen is also Assistant Professor of Neuroscience and of Biophysics at The Johns Hopkins University School of Medicine.

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V. STRUCTURAL BIOLOGY

V. PROGRAM IN STRUCTURAL BIOLOGY

The Program in Structural Biology, initiated in 1986, is the most recently established of the five HHMI research areas. Investigators in this program are located at The Johns Hopkins University, Harvard College, the University of Texas Southwestern Medical Center at Dallas, Baylor College of Medicine, Yale University, Columbia University, and the University of California at San Francisco. These investigators are examining the physical structure and organization of biologic materials through the techniques of x-ray crystallography, electron and optical imaging, nuclear magnetic resonance spectroscopy, and advanced computerized three-dimensional image reconstruction. The Institute is also supporting the development of a new beam line at the synchrotron at the Brookhaven National Laboratory, which will serve as a resource for the larger biomedical community, as well as for investigators within HHMI.

The research of Assistant Investigator Axel T. Brünger, Ph.D. (Yale University) and his colleagues is at the interface between theory and experiment in the area of structural biophysics. Their research tools are simulation methods of computational chemistry, adapted to the requirements of macromolecular systems. Their current research effort centers on the development and applications of macromolecular structure determination and refinement, based on x-ray crystallographic or nuclear magnetic resonance (NMR) spectroscopic data. Structures of the influenza virus hemagglutinin with altered receptor binding complexed with cellular receptor analogues have been refined by simulated annealing, and a new search strategy based on Patterson correlation refinement has been developed to obtain initial phases for single-crystal diffraction data by molecular replacement.

The work of Investigator Wayne A. Hendrickson, Ph.D. (Columbia University) and his colleagues features a mix of crystallographic methodology development and the applications of this technology to biologically significant problems. Of particular importance in the past year has been the further development of general methods for introducing appropriate anomalous scattering centers for direct structural analysis by the multiwavelength anomalous diffraction (MAD) method, using synchrotron radiation. Selenomethionyl proteins produced in recombinant systems and synthetic brominated nucleic acids serve well in this regard. These techniques have been used in the structural analysis of a bromoDNA/drug complex and of interleukin-1α. MAD analyses on selenomethionyl thioredoxin and ribonuclease H are also well advanced.

The laboratory of Associate Investigator David A. Agard, Ph.D. (University of California at San Francisco) focuses on understanding the relationship between structure and function at the cellular and molecular levels. In a collaboration with the laboratory of Investigator John W. Sedat, Ph.D. (University of California at San Francisco), Dr. Agard is using novel three-dimensional imaging methods to investigate the structure of mitotic chromosomes and to examine their spatial and temporal behavior throughout the mitotic cell cycle. At a molecular level, Dr. Agard's group is using a combination of site-directed mutagenesis, solution kinetics, and x-ray crystallography to probe the structural basis of enzyme specificity, using the bacterial serine protease α-lytic protease as a model system. Biochemical, genetic, and structural approaches are being used to investigate the unusual folding pathway of α-lytic protease, since it provides a unique opportunity for studying a stable folding intermediate and the "foldase" that converts it to the mature, active enzyme. Determination of the crystal structure of apolipoprotein E (apoE), an important protein in human cholesterol metabolism, is also being pursued.

Dr. Sedat's efforts are directed at reaching a structural understanding of the interphase chromosomes in the eukaryotic nucleus through an approach that is at the limits of optical and electron microscopic resolution for three-dimensional analysis. *Drosophila* is used as a genetically and developmentally defined model organism. Dr. Sedat's group has developed and applied real-time three-dimensional optical microscopy for living samples, three-dimensional structure analysis of the distribution of the nuclear envelope protein lamin, and three-dimensional *in situ* hybridization of specific DNA probes.

Investigator Johann Deisenhofer, Ph.D. (University of Texas Southwestern Medical Center at Dallas) and his colleagues have continued their work on aspects of the three-dimensional structure of a large pigment-protein complex, the photosynthetic reaction center from a purple bacterium. Sitting in the cell membrane of *Rhodopseudomonas viridis*, this molecule performs the first steps in photosynthesis, the conversion of light energy into chemical energy. Knowledge of this structure has already led to a better understanding of photosynthesis.

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The laboratory of Assistant Investigator Robert O. Fox, Ph.D. (Yale University) has investigated the role of the amino acid sequence in determining the folding pathways and the final, detailed three-dimensional structure of globular protein molecules. The laboratory has combined the methods of x-ray crystallography, NMR spectroscopy, and molecular genetics to investigate how the sequence determines turn and loop structures in immunoglobulin molecules and certain enzyme molecules, such as staphylococcal nuclease. In a genetic analysis of a β-turn in the nuclease, point mutations have been identified that transform a β-turn structure from one ideal type to another, while maintaining the globular protein structure. Thus a β-turn element was transplanted from one protein to another, maintaining the local three-dimensional structure of the turn. The crystal structure of a Fab fragment has also been solved; this has suggested a structural mechanism by which somatic mutations may modulate Fab-hapten affinity.

The laboratory of Investigator Don C. Wiley, Ph.D. (Harvard College) continues to study the structure and function of cell and viral surface molecules. They have determined the structure of a complex between an influenza viral protein and a component of its cellular receptor. This should facilitate the design of drugs to inhibit the virus from binding to cells. Using recombinant DNA methods, Dr. Wiley and his colleagues have also studied the membrane fusion mechanism by which viruses enter cells. Other studies determined the three-dimensional structure of the human histocompatibility antigen, which is 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 being studied.

The research of Investigator Stephen C. Harrison, Ph.D. (Harvard College) and his colleagues involves the elucidation of the detailed three-dimensional molecular structures of assemblies such as viruses, protein/DNA complexes, and cell-surface receptors. Such structural information is essential for understanding how viruses assemble, how the expression of genes is selectively activated or repressed, and how viruses and other ligands are taken up by cells. During the last year the structure of SV40, a DNA tumor virus, has been determined. This is the largest virus to be visualized in such detail. Progress has also been made in understanding how regulatory proteins of two different types recognize their specific DNA-binding sites.

The laboratory of Assistant Investigator Sherry L. Mowbray, Ph.D. (University of Texas Southwestern Medical Center at Dallas) is studying receptors and membrane proteins. Mutants of a membrane protein for bacterial chemotaxis have been used to explore models for transmembrane signaling. Three soluble receptors for chemotaxis and transport are being studied by x-ray crystallography, with the aim of learning about the activation of receptor proteins and the transfer of signal information between proteins. The leader peptidase of *Escherichia coli* is also being studied to help outline the events involved in the transfer of proteins across membranes.

The research of Associate Investigator Carl O. Pabo, Ph.D. (The Johns Hopkins University) and his colleagues has focused on the structure and design of proteins that regulate gene expression. The laboratory is attempting to understand how proteins recognize specific sites on double-stranded DNA and how the bound proteins regulate gene expression. Dr. Pabo's research has used a high-resolution crystal structure of the λ repressor-operator complex as the basis for continued genetic and structural analysis of repressor-operator interactions. Crystallographic studies of other repressors and repressor-operator complexes are in progress, and software is being developed for the general analysis and design of DNA-binding proteins.

In the laboratory of Investigator Florante A. Quiocho, Ph.D. (Baylor College of Medicine), structural analysis continues to reveal basic features of protein-ligand interactions that are fundamental to biological specificity and function. A 3.5 Å resolution electron density of the enzyme adenosine deaminase has been calculated, providing an initial look at the structure of this key enzyme in normal immune system development. Site-directed mutagenesis and binding studies, coupled with x-ray analyses of binding proteins, provide insight into a variety of ligand recognitions. Crystals of an antibody against gp120, the coat protein of the human immunodeficiency virus (HIV), are of scientific and medical interest, as they promise to illuminate the structure of a possible antibody-binding site on the AIDS virus. The well-refined high-resolution structure of calmodulin allows understanding of its interactions with calcium and its target enzymes.

Protein molecules act both as regulators and targets of regulation in biological processes. The laboratory of Assistant Investigator Stephen R. Sprang, Ph.D. (University of Texas Southwestern Medical Center at Dallas) has focused on the allosteric enzyme glycogen phosphorylase, which is regulated

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by phosphorylation, metabolites, and subunit cooperativity. More recently the laboratory has begun structural studies of G transduction proteins that couple the β-adrenergic receptor to regulation of adenylate cyclase, thus initiating the cascade of phosphorylation events that results in the activation of phosphorylase and many other enzymes. Structural studies have been initiated of cachectin, or tumor necrosis factor, which binds to a cellular receptor and initiates a series of complex metabolic changes characteristic of shock and inflammation. Studies of a new class of membrane-associated calcium-binding proteins that may play a significant role in membrane morphogenesis are also under way.

The aim of the research of Investigator Paul B. Sigler, M.D., Ph.D. (Yale University) and his colleagues is to understand cellular regulatory mechanisms in detailed chemical terms. Two processes receive special attention: 1) transcription of the genetic message and 2) transduction of external signals across the cell's membrane. Current research concerns the recognition by a regulator protein of genetic signals in the DNA, and the enzymatic mechanism of "second messenger" release. High-resolution x-ray crystallography is used to visualize, in detail, the molecular structures involved. From these structures, chemical mechanisms can be inferred and then checked, using biochemical and genetic methods.

The general goal of the laboratory of Investigator Thomas A. Steitz, Ph.D. (Yale University) has been to understand the biological function of macromolecules in terms of their detailed molecular structure. The work addresses the following questions 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? The specific systems under study include the catabolite gene activator protein, the Klenow fragment of DNA polymerase I (in collaboration with Dr. Catherine Joyce), resolvase, *rec*A, and $tRNA^{Gln}$-synthetase complex. To understand enzyme mechanisms, Dr. Steitz and his colleagues are using site-directed mutagenesis to determine the effect of specific mutations on the three-dimensional structure of the protein and its complexes with substrates.

STRUCTURAL BASIS FOR ENZYME SPECIFICITY AND CHROMOSOME STRUCTURE

DAVID A. AGARD, PH.D., *Associate Investigator*

Dr. Agard's research is 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 actively pursued: 1) three-dimensional analysis of diploid chromosome structure and topology; 2) studies on the structural determinants of specificity, using α-lytic protease as a model system; 3) functional and structural analysis of the role of the precursor in proper folding of α-lytic protease; and 4) pursuit of the first three-dimensional crystal structure of apolipoprotein E (apoE), an important protein in human cholesterol metabolism.

I. Three-dimensional Analysis of Chromosome Structure.

The Agard laboratory is engaged in a close collaboration with Dr. John W. Sedat (HHMI, University of California at San Francisco). The primary aim of their research is to provide a physical basis for understanding chromosome behavior and function by directly determining the three-dimensional structure of eukaryotic chromosomes as a function of both transcriptional state and the cell cycle stage.

A. *Three-dimensional imaging.* The Agard and Sedat groups have developed the necessary technologies (hardware and software) to allow them to examine complex noncrystalline specimens in three dimensions, using electron microscopy (EM) and light microscopy. Three-dimensional EM analysis is performed using electron microscopic tomography, which allows one to look inside chromosomes (or other cellular structures) and examine their internal arrangements in three dimensions at ~50–100 Å resolution. The HHMI intermediate voltage electron microscope at the University of California at San Francisco is equipped with an ultrahigh angle tilt stage and a cooled charge-coupled device (CCD) imager. This provides ideal imaging capabilities for EM tomography of specimens up to 0.5 μm thick. Improved software for aligning images, merging data in Fourier space, and calculating the reconstructions is now under development. Three-dimensional light microscopic reconstructions use high-resolution optical sectioning microscopy, a cooled CCD detector, and image processing to remove out-of-focus information. Powerful software for the display, analysis, and model building that preserves all of the three-dimensional gray-level information has also been developed and continues to be improved. Most exciting is the development of the capability to record time-lapse three-dimensional data on living embryos, thus making possible the detailed study of complex dynamic cellular phenomena.

Technological advances have been driven by the needs posed by the biological problems, and not simply by the desire for better technology. This is most evident in the balanced developmental effort for hardware and software for data collection, data processing, display, and analysis, as all of these must work together for the biology to succeed.

B. *Higher order chromosome structure.* Dr. Agard and his colleagues have made significant progress in analyzing the details of higher order chromosome structure. Buffer conditions have been found that preserve the 250 Å chromatin fibers but allow the overall degree of condensation to be varied. Careful comparisons of chromosome and interphase nuclear morphology, using three-dimensional fluorescence microscopy with living and permeabilized cells, has indicated the conditions most similar to the *in vivo* state. From EM studies it has been learned that both the radial-loop and sequential helical coiling models of chromosome structure are gross oversimplifications. Mitotic chromosomes are built from a fundamental nucleosomal fiber of ~110 Å diameter that is organized into higher order structures measuring ~250, 600, and 1,300 Å.

The past year has been spent in optimizing conditions for preserving the highest level of discrete chromatin organization: the 1,300 Å fiber. There is a narrow window in telophase that is particularly favorable for viewing this structure. A very high resolution three-dimensional data set comprising ~150 tilted views over a ±75° range has been collected, using the newly developed CCD detector and ultrahigh tilt stage. Although the reconstruction has only recently been obtained, the improvement in quality compared with previous reconstructions using film data is extraordinary. Within regions of the reconstruction it should be possible to trace the individual 110 Å fibers within the 250 Å fiber, to trace the path of the 250 Å fibers within

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the 600 Å structures, and to determine how 600 Å structures pack to form the final 1,300 Å structure. This new level of clarity is undoubtedly due in part to the use of the cooled CCD to record the tilt data directly and digitally. Current efforts focus on interpreting this reconstruction, recording more data, and improving data collection, reconstruction, and display.

C. *Three-dimensional optical microscopy and chromosome structure.* The three-dimensional optical microscopic studies pioneered by Drs. Agard and Sedat have been used as a powerful control for EM sample preparation methods and also to investigate the dynamic behavior of chromosomes throughout the cell cycle. Three-dimensional resolution in the light microscope is now sufficient to trace the path of chromosomes in diploid nuclei from early prophase through late telophase. The most exciting results have come from a combination of three-dimensional *in vivo* microscopy with the higher resolution data available from fixed specimens. These data suggest that the complex process of chromosome condensation is actually nucleated at discrete sites on the nuclear envelope. Furthermore, chromosome compaction that occurs during the prophase-metaphase transition begins at the centromere and spreads in a wave-like manner out to the teleomeres. This work has led to the first reliable data on the three-dimensional spatial arrangement of diploid chromosomes within the nucleus. Recently developed three-dimensional *in situ* hybridization methods will be used to determine what sequences lie at the nucleation points.

D. *Use of antibodies to combine structure with function.* Staining of *Drosophila* nuclear division cycle 14 embryos with an antilamin monoclonal antibody produces a noncontinuous staining pattern of interlocking fibers in which a significant fraction of the nuclear surface is not occupied by lamins. Similar staining patterns have been observed in HeLa and *Drosophila* Kc cells. These unanticipated patterns suggest that the extremely large oocytes may be anomalous. Analysis of the spatial distribution of topoisomerase II staining indicates that at high resolution, topoisomerase II is not colocalized with the chromosomes but instead resides largely on structures that seem to wind around the chromosomes. Although still preliminary, this result has striking implications for models of chromosome and nuclear matrix structure.

II. Structural Basis of Enzyme Specificity.

One fundamental function of an enzyme is to be specific, that is, to limit the number of substrates on which it can act. Since catalysis derives from the enzyme's ability to stabilize the transition state of a reaction, specificity is a consequence of selective binding of the transition states for preferred substrates. Dr. Agard has chosen α-lytic protease as an ideal model system to investigate structural and energetic aspects of specificity. In addition to there being a wide range of substrates and inhibitors available, its binding pocket is made of the side chains of three amino acids (Met192, Met213, Val217A), providing a large volume that could be experimentally manipulated.

The enzyme from the soil bacterium *Lysobacter enzymogenes* has been cloned and expressed at usable levels in *Escherichia coli* (100–200 mg/10l fermenter run). Through a collaboration with Dr. Charles Kettner (DuPont), Dr. Agard and his colleagues have been able to obtain a large number of tight-binding peptide boronic acid inhibitors. These inhibitors were used to determine high-resolution crystal structures for more than a dozen of the inhibitor-enzyme complexes using the native enzyme. These structures have provided insights into the structure of the transition state and the importance of substrate hydrogen bonding for its stabilization, as well as basic information on steric exclusion and specificity.

By mutation, Dr. Agard and his co-workers have been able to alter dramatically the pattern of substrate specificity while maintaining or increasing enzyme activity (activity toward Phe substrates was increased by nearly six orders of magnitude). Structural analyses of the first few mutants as free enzymes and as complexes have provided insights into the mechanism of specificity and indicated the crucial role that protein flexibility plays in selectivity. Current efforts involve mapping the detailed energetics of protein flexibility through further mutagenesis, kinetic, and crystallographic analyses. More than 25 structures have been determined. A collaboration with Dr. Peter Kollman (University of California at San Francisco) has been initiated to apply theoretical methods to this complex problem.

III. Structural and Biochemical Probes of Folding of α-Lytic Protease.

An unexpected benefit of choosing α-lytic protease was revealed when the gene was cloned and

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found to be synthesized as a prepro-enzyme. Subsequent experiments in *E. coli* have demonstrated that the 166–amino acid pro domain is required for the proper folding of the 198–amino acid protease domain. Recent experiments indicate that covalent attachment of the pro and protease regions is not required—coexpression of separate pro and protease domains leads to active protease. This folding in trans can also be accomplished by mixing cellular extracts that separately contain the precursor and mature domains. The current thinking is that the temperature stability and protease resistance of the mature protease are generated at the expense of ease of folding. That is, there is a high-energy barrier between the folded and unfolded states that is too high for the mature protease to cross by itself. The precursor acts as a "foldase" to stabilize the transition state for folding; it essentially acts as a template on which the mature enzyme finds its active conformation.

Current work aims at genetically dissecting the precursor and purifying sufficient misfolded mature domain and pro-enzyme for crystallization. Pro-enzyme has been partially purified under denaturing conditions and can be refolded *in vitro*. This provides the unique opportunity to study the detailed structure of a folding intermediate and the "enzyme" that catalyzes the transition to the final folded state.

IV. Structure of Apolipoprotein E.

ApoE is an important protein in cholesterol metabolism in mammals. It is a component of several classes of circulating plasma lipoprotein complexes, being especially abundant in chylomicrons, very low density lipoproteins (VLDL), and certain high-density lipoproteins (HDL). These particles function in intravascular lipid transport, which involves cellular uptake of lipoproteins via specific, apolipoprotein-mediated binding to a cell surface receptor (the LDL or apoB–E receptor). ApoE is one of two proteins that can bind to the LDL receptor and thus has a major role in triglyceride and cholesterol metabolism. The protein itself has two distinct structural and functional domains: the amino-terminal 22 kDa domain contains the receptor binding functionality, whereas lipid binding resides primarily with the 10 kDa carboxyl-terminal domain.

In collaboration with the Mahley group (Gladstone Foundation Laboratories for Cardiovascular Disease), Dr. Agard has obtained crystals of the 22 kDa receptor-binding domain that are suitable for high-resolution x-ray analysis, $P2_12_12_1$, which diffract beyond 2.5 Å. They have collected low-resolution native data and are currently screening for derivatives. High-resolution data have been obtained at –150°C and are being processed. Future work will involve structural analysis of mutants of this protein that are known to cause serious human cholesterol disorders.

Dr. Agard is also Associate Professor in the Departments of Biochemistry and Pharmaceutical Chemistry at the University of California at San Francisco.

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CRYSTALLOGRAPHIC PHASING AND REFINEMENT

AXEL T. BRÜNGER, PH.D., *Assistant Investigator*

Dr. Brünger's research is focused on the interface between theory and experiment in the area of structural biophysics. The research tools are simulation methods of computational chemistry adapted to the requirements of macromolecular systems. The current effort centers on development and applications of macromolecular structure determination and refinement based on x-ray crystallographic or nuclear magnetic resonance (NMR) spectroscopic data.

I. Refinement of the Influenza Virus Hemagglutinin by Simulated Annealing.

The hemagglutinin (HA) glycoprotein of the influenza virus membrane mediates the receptor-binding and membrane fusion activities required for entry of the virus into a host cell and is also the primary antigen of the virus. The HA is a homotrimer consisting of a large ectodomain, a small transmembrane region, and a small domain inside the virus. Each monomer consists of two disulfide linked chains, HA_1 and HA_2, formed by post-translational cleavage of a single polypeptide precursor; the carboxyl-terminal region of HA_2 anchors the HA in the membrane. Treatment of the X:31 virus with the protease bromelain produces the trimeric water-soluble ectodomain BHA (molecular weight ~210 kDa). Structures of mutant BHAs with altered receptor binding and complexes of BHA with cellular receptor analogues have been obtained in Dr. Don C. Wiley's laboratory (HHMI, Harvard College). Despite the 3 Å resolution limit imposed by the BHA crystals, there is great interest in using these structures for modeling studies, as part of an effort to design anti-influenza drugs. In collaboration with Dr. Wiley, these structures were refined, using Dr. Brünger's method of crystallographic refinement by simulated annealing, with the goal of obtaining models as close to idealized as possible.

The mutant structure G146D with the best diffraction data was refined first as a monomer, using a reciprocal space method for averaging the x-ray structure factor derivatives over the threefold noncrystallographic symmetry, and a nonbonded energy term describing the interactions of the monomer with its trimer symmetry mates. Subsequently the entire trimer was refined to model lattice interactions properly, using positional and isotropic temperature factor noncrystallographic symmetry restraints in those portions of the molecule not involved in lattice contacts. This structure was then used as the basis for refinement of the other three crystallographically isomorphous HA mutant structures, L226Q, L226Qs, and D1112Gs, where the latter two structures are complexed with influenza virus receptor, sialic acid.

The refinements accomplished the goals of obtaining HA models of low R factor and favorable empirical energies for modeling or analogue drug design purposes. The sialic acid complexes obtained in this work have significantly better geometry than the original coordinates reported previously. Refinement by simulated annealing required little manual intervention. However, this refinement is not fully automatic; there were always some regions of the structures that required manual adjustment. Overall, however, the process worked well at idealizing a relatively low resolution crystal structure with a minimum of manual intervention. The refined coordinates of G146D, L226Q, L226Qs, and D1112Gs have been deposited in the Brookhaven data bank.

II. Extension of Molecular Replacement: A New Search Strategy Based on Patterson Correlation Refinement.

In macromolecular crystallography, the initial determination of phases by molecular replacement (MR) is often attempted if the structure of a similar or homologous macromolecule is known (search model). MR involves the placement (i.e., rotation and translation) of the search model in the unit cell of the target crystal to obtain the best agreement between calculated and observed diffraction data. The optimally placed search model is used to obtain initial phases for structure building and refinement. This approach may or may not succeed; many successful cases reported involve search models with a backbone atomic root-mean-square difference of <1 Å from the target structure.

Recent progress in obtaining approximate three-dimensional models of macromolecules from information other than diffraction data suggests an increased use of MR to solve crystal structures. For instance, the database of known protein sequences and protein structures is growing rapidly. Techniques for aligning sequences, such as consensus

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templates, have been developed to recognize distantly related proteins or protein domains and to carry out model building on the basis of the known protein structures. Another example is the determination of three-dimensional structures of small proteins and nucleic acids from NMR NOESY (nuclear Overhauser enhancement two-dimensional spectroscopy) experiments. The search models obtained by these methods are often approximate. For example, the atomic root-mean-square differences in the protein core of homologous proteins range from 0.76 to 2.3 Å. Thus MR could become difficult, if not impossible. It is therefore desirable to extend the applicability of MR.

If there is one molecule in the crystallographic asymmetric unit, then three positional and three angular parameters fully describe the placement of the search model in the unit cell of the target crystal. This six-dimensional search can be reduced to a sequence of a three-dimensional angular search using a rotation function (RF), followed by a three-dimensional positional search using a translation function (TF). This procedure assumes that the highest peak of the RF yields the correct orientation. Examples are known where this is not true. Due to advances in computer technology, multidimensional search strategies with more than three parameters are now possible.

Even six-dimensional searches may fail to solve the crystal structure. In this case, Dr. Brünger proposes to vary the atomic coordinates of the search model in a neighborhood of the initial positions. For instance, R factor refinements could be carried out with the search model placed in the most likely orientations and positions, as determined by a multidimensional search. However, this procedure would be computationally intensive, since the translation searches may not yield a unique solution, and one would have to carry out refinements for several peaks for the translation search for each selected orientation.

A new method was developed to refine atomic coordinates of the search model *prior* to translation searches. The target function for the new refinement method, PC refinement, consists of a Patterson energy term combined with an empirical energy function. The Patterson energy term is proportional to the negative correlation coefficient PC between the squared amplitudes of the observed and the calculated normalized structure factors. The normalized structure factors are computed with the search model placed in a triclinic unit cell identical in geometry to that of the crystal. The empirical energy function represents information about the geometry and nonbonded interactions of the macromolecule. PC refinement of individual atomic coordinates may be impractical for large molecules because of the large computational expense. In this case, generalized coordinates, such as the orientation and position of rigid groups, can be refined against a target function that simply consists of the Patterson energy term without an empirical energy function.

A combined MR and PC-refinement strategy was proposed in Dr. Brünger's laboratory. First, a conventional RF is carried out. All sampled orientations are sorted with respect to the RF value, and a large number of the highest peaks are selected for PC refinements. Finally the PC-refined search models with the highest correlation coefficients are used for conventional translation searches.

Computer studies were carried out that were aimed at evaluating the utility of PC refinement. A particular search model of crambin with a 2 Å backbone atomic root-mean-square difference from the crystal structure failed to provide the correct orientation when using an RF or a six-dimensional search. PC refinements of the search model in the most likely orientations resulted in the correct orientation having the lowest value of the target function. This enabled Dr. Brünger and his colleagues to solve the crambin structure, starting with the PC-refined search model. In an application to myoglobin, it was shown that rigid-group PC refinement of the eight α-helices has an approximate radius of convergence of 13° for orientation parameters. A search model of myoglobin with the α-helices artificially tilted by 13° failed to provide the correct orientation when an RF or a six-dimensional search was used. PC refinements uniquely determined the correct overall orientation of the myoglobin search model by returning the α-helices to their original placements.

Dr. Brünger is also Assistant Professor of Molecular Biophysics and Biochemistry at Yale 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 with the methods of x-ray crystallography. The aim of these studies is to understand folding, structural stability, and function of macromolecules. Of particular interest are protein-protein interactions, the structure of membrane-spanning and membrane-associated proteins, photochemical energy conversion, energy transfer, and electron transfer. An ideal system to study all these aspects of structure and function has been the photosynthetic reaction center (RC) from the purple bacterium *Rhodopseudomonas viridis*. Experiments to extend structural studies to other systems are under way.

I. Reaction Center from *Rhodopseudomonas viridis*.

The RC was the first integral membrane protein whose structure was determined to atomic resolution. It is a complex of four protein subunits (cytochrome, L, M, and H) of about 300 amino acids each. Associated with these protein subunits are 14 cofactors: 4 hemes, 4 bacteriochlorophyll-*b*, 2 bacteriopheophytin-*b*, 2 quinones, 1 nonheme iron, and 1 carotenoid. The structure and arrangement of the subunits L and M and of their associated cofactors show a high degree of local twofold symmetry.

The RC performs the first steps of the conversion of light energy into chemical energy during bacterial photosynthesis: a photon is absorbed by the "special pair," a closely associated pair of bacteriochlorophylls. From the excited special pair an electron is transferred to one of the bacteriopheophytins within ~3 ps. From there the electron is passed on within ~200 ps to the quinone Qa. The electron ends up at the second quinone, Qb. While being transferred from the special pair to Qa, the electron crosses the membrane bilayer. Qb picks up two electrons and two protons and dissociates from the RC. Additional molecular systems in the bacterial membrane transfer electrons and protons back through the membrane; the electrons are recycled to the RC, and the protons build up a gradient that can be used by the bacterial cell to produce, for example, ATP.

Crystallographic refinement of the atomic model of the RC at 2.3 Å resolution has been completed; the R value, computed from 10,288 model atoms (including 201 waters) and from 95,000 unique reflections is 0.193. The refinement of the RC structure was done in collaboration with Drs. Hartmut Michel and Irmgard Sinning (Max-Planck-Institut für Biophysik, Frankfurt, FRG) and with Dr. Otto Epp (Max-Planck-Institut für Biochemie, Martinsried, FRG).

A. *Visualization of the detergent micelles.* Before crystallization the RC molecules had to be solubilized, using the detergent *N,N*-dimethyldodecylamine-*N*-oxide (LDAO). Crystallization had been carried out in the presence of LDAO and of the small amphiphile heptane-1,2,3-triol. These molecules turned out to be disordered in the crystal and therefore could not be located using x-ray diffraction methods (an exception is one completely ordered LDAO molecule per RC).

To determine the distribution of the detergent in the crystal, a neutron diffraction study at 15 Å resolution was carried out. The method of contrast variation, using different ratios of H_2O and D_2O in the crystal mother liquor, led to a clear picture of detergent micelles formed around the central part of the RC molecule, where the protein surface is highly hydrophobic. This confirmed the assumptions, based on structural and functional considerations, of the position of the RC in the membrane. It is also the first successful visualization of a detergent micelle in a membrane protein crystal. This work was done in collaboration with Dr. Michel and Drs. Michel Roth and Anita Lewit-Bentley (Grenoble, France).

B. *Search for structural changes.* One question that could not be answered by the structure analysis of the native RC is whether the complex undergoes structural changes during the various stages of light absorption and electron transfer. Experiments to answer this question were done by treatment of RC crystals with oxidizing (potassium ferricyanide) and reducing (ascorbate) agents and collection of sets of x-ray diffraction intensities. Refinement against these data, starting from the refined native model, did not show any significant structural changes.

To achieve conditions closer to the ones under which the RC works *in vivo*, attempts are being made to measure x-ray data from crystals under illu-

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mination (in collaboration with Susan Buchanan and Drs. Sinning and Michel). Crystals with reconstituted Qb are being used for these experiments. A synchrotron x-ray source is necessary to provide the high primary beam intensity needed for this work.

C. *Herbicide-resistant mutants.* One indication of the structural similarity between RCs from purple bacteria and from plant photosystem II is the sensitivity of both these RCs to the same herbicides. Herbicide-resistant RC mutants can be obtained by culturing the bacteria in the presence of herbicide. X-ray diffraction experiments using the difference-Fourier technique are expected to indicate the structural changes and thereby to increase understanding of the mechanism of herbicide resistance (in collaboration with Drs. Sinning and Michel).

II. New Projects.

Structural studies on several other proteins in other biological systems have been started, to investigate various aspects of the RC structure, such as membrane protein structure, electron transfer, and protein-protein interactions. All these studies are currently in the stage of crystallization experiments.

Dr. Deisenhofer is also Regental Professor 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., *Assistant Investigator*

Dr. Fox is investigating the role of the amino acid sequence in determining the folding pathways and the final detailed three-dimensional structure of globular protein molecules. One current focus is the role of sequence in determining turn and loop structures at the combining sites of immunoglobulin molecules and the active sites of enzymes such as staphylococcal nuclease. These investigations should shed light on the structural basis of immunoglobulin maturation and provide guidelines for the engineering of new protein molecules.

I. Structural Basis of Immunoglobulin Maturation.

The sequence diversity found in immunoglobulin molecules is generated at several different levels. Combinatorial variability arises from the rearrangement of germline V, D, and J gene segments as B cells develop. Somatic mutation of the variable region of the immunoglobulin genes is stimulated when a B cell recognizes an antigen, adding further diversity to the immune response. Although the generation of antibody sequence diversity by these mechanisms has been well characterized, the structural basis by which sequence differences modify antibody affinities remains relatively unexplored.

A panel of 12 monoclonal antibodies to a TEMPO-dinitrophenyl hapten have been prepared, and the heavy- and light-chain cDNAs have been sequenced by Dr. Harden McConnell's laboratory (Stanford University). Three members of this panel, AN01, AN02, and AN03, use the same or a similar set of germline genes, although they are the result of different VJ and VDJ rearrangements. The AN02 Fab binds the hapten more tightly than the AN01 or AN03 Fab molecules. Sequence analysis of the AN02 germline genes for AN02 (at Stanford) indicates several changes occurred in the variable regions of the heavy and light chains as the immune response matured. Expression of the germline sequences, and the AN01–AN12 panel of monoclonal antibodies will provide an opportunity to investigate the structural role of VJ and VDJ rearrangement and somatic mutation in determining antibody-hapten affinity.

The Fab fragment of the monoclonal antibody AN02 has been crystallized, with and without bound hapten. The Fab-hapten complex crystal structure has been solved by molecular replacement in collaboration with Dr. Axel T. Brünger (HHMI, Yale University), using the PC-refinement methods developed in his laboratory. The structure has been refined to 2.8 Å resolution, revealing the Fab-hapten interactions in atomic detail. Refinement at the diffraction limit of this crystal form (2.0 Å) will provide a more detailed view of the Fab-hapten complex. Analysis of the sequence differences between the germline and mature AN02 genes in light of the crystal structure suggests several structural mechanisms by which Fab-hapten affinity may be modified.

II. Constraining β-Turn Conformation in Model Immunogens.

Antipeptide antibodies raised against a short segment of a known protein sequence have proven in many cases to be highly specific biological probes. A limitation of the technique is that the resulting antibody displays a low binding affinity for the protein antigen, which precludes its use in applications that require a strong antigen-antibody interaction. The low affinity is thought to be due to the conformationally unconstrained peptide immunogen, which can stimulate B cell clones to produce antibodies against nonnative peptide structures. To improve epitope presentation, exposed turn sequences have been selected from known protein structures and the conformation of the sequence constrained by substitution into a turn site of a host protein. Incorporation into a stable protein should restrict the guest turn sequence to a limited conformational range that better mimics the structure of the parent molecule.

The first sequence selected is an exposed five-residue loop from concanavalin A. The sequence was substituted for four turn residues in staphylococcal nuclease. The resulting hybrid protein adopts a stable folded conformation and retains nuclease enzymatic activity. The crystal structure of the hybrid has been determined to 1.8 Å resolution, revealing that the conformation of the sequence in concanavalin A has been preserved when introduced into a foreign turn site. The modest stability of the original hybrid protein can be returned to near wild-type levels by limiting the substitutions to surface residues.

Attempts to raise monoclonal antibodies to the hybrid protein or a linear peptide containing the

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concanavalin A sequence have failed to produce an IgG response that recognizes concanavalin A. Other turn sequences have been selected from known structures, with the additional criteria that the turn is known to be immunogenic. The important finding from this first design of a hybrid immunogen is that the conformation of the concanavalin A β-turn was successfully transferred to a host protein in a native conformation.

III. Genetic Analysis of a β-Turn.

A genetic system has been developed 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). An M13mp18 construct has been prepared that allows an oligodeoxynucleotide mixture to be cloned into the nuclease gene, which has a random distribution of bases over codons 27–31. This oligonucleotide mixture was cloned into the vector to produce a library of nuclease mutants, each with a unique sequence at the β-turn site. Clones that displayed enzymatic activity on assay plates (~3–8%) were selected for DNA sequencing. Analysis of 130 β-turn sequences from active nucleases indicates strong preferences for and against particular amino acids at each of the five positions. A further mutagenesis experiment, varying only the central two β-turn residues (28–29), resulted in a high proportion of enzymatically active nuclease variants. A denaturant-dependent activity assay has been developed to estimate the impact of these substitutions on the stability of the nuclease variants. A series of "intra-turn" complementation mutants indicate structural interactions between sites within the β-turn. Rules for β-turn formation may be derived from this experimental approach. A determination of the influence of these β-turn substitutions on the thermodynamic stability of pure protein variants is in progress.

IV. 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 nuclear magnetic resonance (NMR) time scale. The interconversion of these folded conformers and the thermal unfolding kinetics have been investigated by magnetization-transfer NMR experiments. 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 proline isomerism hypothesis is also supported by variant proteins prepared by site-directed mutagenesis, where proline 117 has been changed to a glycine (P117G) or a threonine (P117T). NMR spectra of these mutants do not indicate the conformational heterogeneity present in the wild-type protein. A number of additional amino acid substitutions in this β-turn region display altered cis-trans equilibrium constants. Thus the free-energy contribution of amino acid residues to the stabilization of type VI β-turns can be evaluated using these methods.

The P117G and P117T nuclease variants have been crystallized both with and without Ca^{2+} and a diphosphonucleotide inhibitor. The structures have been solved and refined at high resolution. The P117G mutation causes residues 115–118 to adopt a type I′ β-turn structure, while the P117T mutation has produced a type I turn geometry.

Studies of how amino acid substitutions in this region affect the equilibrium and kinetics of the isomerization, and the resulting crystal structures, will lead to a better understanding of the forces that stabilize protein molecules.

V. *De Novo* Enzyme Design.

Dr. Fox and his colleagues are working toward the total design of a folded protein structure that should hydrolyze amino acid esters based on an α + β backbone fold. The approach has been to select a protein fold and active-site geometry and then define an amino acid sequence that will favor that fold. Cycles of design, peptide synthesis, and detailed physical and structural analysis, followed by redesign of protein substructures and the final fold, should provide insight into the factors involved in stabilizing folded protein structures and in catalysis. A stable amphipathic α-helix has been designed as an independent element of the final structure. Studies are in progress to define the β-sheet component of the protein, using two stranded β-hairpin model systems.

Dr. Fox is also Associate Professor of Molecular Biophysics and Biochemistry at Yale University.

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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 targeted three broad areas for x-ray crystallographic analysis of assembly and recognition: viruses and their interactions with cells, protein/nucleic-acid complexes, and cell-surface receptors.

I. Viruses.

The structure of SV40, one of the simplest of the double-stranded DNA viruses, has been determined at 3.8 Å resolution. This is the largest of the icosahedral viruses to be visualized in such detail, and its structure differs in important ways from the common design seen in various positive-strand RNA viruses. The polyomaviruses, of which SV40 is an example, are composed of 360 copies of an ~45 kDa protein, VP1; ~30–60 copies each of two minor proteins, VP2 and VP3; and a closed, circular DNA chromosome complexed with histones. The subunits of VP1 form pentamers; 72 such pentamers pack into the icosahedrally symmetric coat. The geometry of this coat requires that identical VP1 pentamers assemble with either five or six neighbors. The pentamers are elongated, roughly cylindrical objects, packing tightly together at a radius of ~200 Å and projecting outward to a radius of ~250 Å.

The VP1 subunit fold is based on two opposed β-sheets with radially directed strands. Each sheet has four principal strands, connected according to the "Swiss roll" pattern found in a number of other proteins, including the subunits of the icosahedral RNA viruses. The sheets are extended by additional strands contributed from neighboring subunits in the VP1 pentamer. Loops connecting the strands extend outward to form the tip of the cylindrical pentamer, and one large loop covers its lateral surface. These loops are the principal loci of variability among sequences of related polyomaviruses. In particular, one of them has an eight-residue insertion in the VP1 of murine polyomavirus, and mutational evidence suggests that this loop forms a sialic acid–binding site. A 6 Å resolution structure of murine polyomavirus, computed using the SV40 map for initial phase determination, shows that this loop must form a shallow pocket at the outer margin of the VP1 pentamer. Polyoma requires sialic acid for initial attachment to cells; SV40 does not. Efforts are in progress to obtain well-ordered crystals of polyoma with a bound sialic acid derivative. The SV40 structure also shows how VP1 pentamers can accommodate either five or six nearest neighbors. Each subunit contains 4 short α-helices, so disposed that they contribute to a palisade of 20 such helices, radially directed around the base of the cylindrical pentamer. Alternative packings of these helices enable adjacent pentamers to pack in distinct, yet specific, ways. The capacity of α-helices to pack with others of somewhat variable sequences may be generally important in assemblies where alternative specific contacts must occur.

The SV40 electron density map reveals part of a subunit of VP2 and/or VP3 bound from within along the axis of the hollow VP1 pentamer. These internal proteins thus appear to form links between the pentameric assembly units of the outer shell and the compact but spatially disordered minichromosome.

II. Protein/Nucleic-Acid Complexes.

Crystals of bacteriophage repressor/operator complexes have offered the first view of how regulatory proteins recognize specific DNA sequences. In temperate bacteriophages of *Escherichia coli*, such as 434 and lambda, the different affinities of the repressor for each of a set of DNA operator sites ensure efficient regulation, and the inversely graded affinities of the Cro protein for the same sites create a sharply controlled binary switch. The binding domain of 434 repressor (R1–69) and 434 Cro have been studied as free proteins and in complex with a series of synthetic operators. The structure of R1–69 in complex with a 20 base pair DNA fragment containing the sequence of operator site O_R1 shows in detail how this representative helix-turn-helix protein interacts with its target. Direct, noncovalent contacts between amino acid side chains and the edges of base pairs in the major groove can account for the principal specificity. Nonpolar interactions (complementary, hydrophobic van der Waals surfaces) are as important as hydrogen bonds. Because the protein imposes a precise conformation on DNA when it binds, the relative ease with which the operator can adopt the required conformation affects its affinity. One way in which DNA can adjust to conformational strain is through propeller twisting of its base pairs. The

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consequent non-coplanarity distorts Watson-Crick hydrogen bonds, but certain sequences permit compensatory bifurcated hydrogen bonding. Such sequences at the center of the 434 operators appear to create particularly good binding sites. The 434 Cro protein imposes a similar but distinct conformation when it binds specifically to DNA. The difference involves a shift in the sugar-phosphate backbone, around a nucleotide critical for the distinction between Cro and repressor specificity, and it apparently results from differences in the identity or conformation of various residues contacting the DNA backbone, some of which lie outside the helix-turn-helix element. Only by taking into account the different DNA conformations imposed by these proteins can their characteristic affinities be explained.

Two proteins representing a different class of DNA-binding domains are now being studied. GAL4 (an activator of transcription in yeast) contains one form of a so-called zinc finger, and TfIIIa (a transcription factor for the 5S RNA gene in *Xenopus*) contains another. Crystals of the GAL4-binding domain in complex with DNA and of the TfIIIa-binding region in complex with 5S RNA have recently been prepared.

III. Receptors.

The transferrin receptor (tfR) is a dimer of 90 kDa transmembrane subunits. It transits an endocytotic pathway involving entry into low-pH endosomal compartments via coated pits and coated vesicles. Digestion of purified tfR with trypsin at neutral pH generates a soluble noncovalent dimer of 70 kDa fragment subunits containing most of the extracellular sequence, including the transferrin-binding sites. Below pH 6, the 70 kDa fragment undergoes a conformational transition, leading to reversible association of the dimers in solution. Nonliganded tfR in coated vesicles appears to undergo a similar association. These properties suggest that intracellular sorting events may involve conformational changes of the ectodomain in endosomal lumina. To explore this and other properties of a "typical" receptor participating in the coated vesicle pathway, Dr. Harrison and his colleagues have prepared crystals of the 70 kDa fragments. The crystals diffract to spacings of 3.5 Å, and the structure determination is at a relatively advanced stage.

Crystallographic studies of CD4, the receptor for human immunodeficiency virus (HIV), are also in progress (in collaboration with scientists at Biogen and at the Dana-Farber Cancer Institute). Forms being studied include the complete extracellular part of the molecule and smaller fragments that still retain HIV-binding activity.

Dr. Harrison is also Professor of Biochemistry and Molecular Biology at Harvard University.

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STRUCTURAL STUDIES ON BIOLOGICAL MACROMOLECULES

WAYNE A. HENDRICKSON, PH.D., *Investigator*

Dr. Hendrickson's laboratory studies macromolecular structure, with an aim toward in-depth understanding of biological activity. Diffraction analysis is the primary research tool, but other theoretical, physical, and biochemical methods are also used. Three broad themes are emphasized: crystallographic studies on molecules of immediate interest; methodology development; and general principles of information, dynamics, and assembly.

I. Oxygen-carrying Proteins.

The proteins that transport oxygen—hemoglobins, hemerythrins, and hemocyanins—are subjects of long-standing interest for structural studies. During the past year advances have been made on a number of problems in this area. First, in collaboration with Dr. Emilia Chiancone (La Sapienza University in Rome), the structure of clam hemoglobin from *Scapharca inaequivalvis* has been analyzed at 2–4 Å resolution in the carbonmonoxy state, and the analysis of crystals in the deoxy state has been initiated. This dimeric hemoglobin features a novel subunit interface with directly communicating hemes apparently responsible for cooperative oxygen binding. Second, in collaboration with Dr. Steven Boxer (Stanford University), the structure of a recombinant mutant of human myoglobin has been determined and refined at 2.8 Å resolution. This mutant (arginine for lysine at residue 45) affects the kinetics of ligand binding, and the structure shows a water-mediated interaction with the distal histidine. Third, the giant (3.8 million Da) erythrocruorin from the earthworm *Lumbricus terrestris* was shown to have D_6 molecular symmetry, and structure analysis is proceeding at 5.5 Å resolution. Finally, excellent crystals of a functional fragment obtained after limited proteolysis of octopus hemocyanin have been produced and characterized. Diffraction extends to 1.8 Å Bragg spacings.

II. Streptavidin.

The extraordinarily high binding affinity of avidin for biotin has generated considerable interest in its relation to biophysical principles of ligand binding and the basis it forms for technological applications. The bacterial analogue streptavidin has proved more suitable than avidin in biotechnology and now also in biophysical studies. The structure of orthorhombic crystals of core streptavidin was first determined as a complex with selenobiotin, which was exploited for phase determination in the diffraction analysis. Subsequently the streptavidin structure in its uncomplexed state was also determined in three crystalline modifications: tetragonal and monoclinic forms and the original orthorhombic form. In addition, the avidity structure has been solved by molecular replacement from the streptavidin model. Studies are under way to explore the structural basis of the avidity of streptavidin for biotin, by examining complexes with biotin analogues and preparing to produce site-directed mutants. The orthorhombic crystal form is especially suitable for the binding studies, and additional refined structures are now available for complexes with desthiobiotin, biocytin, and a carboxybiotin derivative. Candidates for directed mutagenesis are being designed on the basis of the structural results, and these will be implemented in an expression system under development in Dr. Charles Cantor's laboratory (Columbia University).

III. New Structural Initiatives.

Studies are also under way on several other structures, most of which have not yet advanced to the level of atomic models. Interest centers on molecules involved in transmembrane signaling, proteins of the immune system, and components of genetic replication and transcription. These projects include a complex of DNA with the drug chromomycin, for which a recently obtained model is being refined against data extending to 1.8 Å spacings; *Escherichia coli* ribonuclease H, which is being analyzed at 1.7 Å resolution (with Dr. Robert Crouch, National Institutes of Health); β-bungarotoxin, for which 2.3 Å data have been processed (with Dr. Paul B. Sigler, HHMI, Yale University); and human insulin, for which 1.8 Å data have been collected. Proteins at early stages of crystallographic analysis include soluble recombinant CD4 (with Dr. Richard Axel, HHMI, Columbia University, and Dr. Ray Sweet at Smith Kline & French) and the carbohydrate recognition domain of a mannose-binding mammalian lectin (with Dr. Kurt Drickamer, Columbia University). Crystals of several other proteins have not yet been fully characterized, and still other projects have not yet yielded crystals.

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IV. Structural Refinement and Protein Dynamics.

The major impediment to an accurate simulation of diffraction data based on macromolecular models from crystallography comes in the description of dynamic properties. Work supported in part by the National Science Foundation is directed at improving descriptions of atomic mobility and thereby advancing methods for structural refinement. Such improvements not only strengthen the reliability of atomic coordinates, but they also characterize significant dynamic features. Atomic mobility parameters from well-refined structures have been correlated with biochemical properties in several instances. The extension of crystallographic refinement methods to two-dimensional nuclear magnetic resonance (NMR) structures is also being examined.

V. The Phase Problem and Anomalous Diffraction.

Diffraction experiments record only the amplitudes for diffracted waves, but both amplitudes and phases are required for a reconstruction of molecular images. The evaluation of these phases presents the major conceptual difficulty in crystallography. Recent theoretical and practical advances in the analysis of multiwavelength anomalous diffraction (MAD) data have borne fruit in a number of applications during the past year. In addition to reports published on earlier applications to lamprey hemoglobin, a bacterial ferredoxin, and streptavidin, new results have also been obtained. These studies have emphasized selenomethionyl proteins and brominated nucleic acids as general phasing vehicles. The structures of selenomethionyl interleukin-1α (with Drs. Marcos Hatada and Brad Graves) and of the complex of chromomycin with d[TU^{Br}GGCCAA] have both been determined from data measured at the Photon Factory in Japan. Analyses of data on selenomethionyl thioredoxin and on selenomethionyl ribonuclease H are also proceeding satisfactorily. Finally, comprehensive measurements have been made for the MAD data associated with copper centers in the octopus hemocyanin fragment. Several advances in the theoretical and computational analysis MAD data have been generated in the course of these applications. This work is supported in part by the National Institutes of Health.

VI. Hughes Synchrotron Resource.

X-ray beam lines for use by HHMI investigators are in the late stages of construction at the National Synchrotron Light Source at Brookhaven National Laboratory. This resource features three beam lines: one with special capabilities for MAD experiments, another dedicated to Laue experiments and position monitoring, and a third for routinely rapid rotation photography and precession characterization. Evacuated beam tubes have been installed for all lines, and "first light" experiments have been conducted. Experimental hutches equipped for BL-2 biosafety containment have been installed on the MAD and rotation lines. Imaging phosphor reader systems have been obtained for efficient x-ray detection, and a facility to house this equipment is under construction.

Dr. Hendrickson is also Professor of Biochemistry and Molecular Biophysics at Columbia University College of Physicians and Surgeons.

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BIOCHEMICAL AND CRYSTALLOGRAPHIC STUDIES OF RECEPTOR AND MEMBRANE PROTEINS

SHERRY L. MOWBRAY, PH.D., *Assistant Investigator*

Dr. Mowbray is using the tools of biochemistry and x-ray crystallography to study the function of receptors and membrane proteins.

I. Receptors in Bacterial Chemotaxis.

Chemotaxis is the process by which motile cells are able to respond to concentration gradients of chemicals in their environment. The genetics and behavior of the system have been well studied in the gram-negative bacteria *Escherichia coli* and *Salmonella typhimurium*. In these cells, two types of receptors, soluble ones from the periplasm and membrane-bound ones from the cytoplasmic membrane, are involved in interpreting and conveying environmental data to the motor machinery of the cell. Some attractants bind to a specific periplasmic protein, which then interacts with the appropriate membrane receptor. Other attractants are capable of binding directly to the membrane receptor proteins. In both cases it is the membrane receptor that carries the information across the cytoplasmic membrane and converts it into usable data for the cell. The membrane receptors are also involved in adaptation to binding signals that persist, through a system that involves covalent modification (methylation) of the internal portions of the membrane receptors.

II. Membrane Receptors.

The relationship between the response systems that utilize periplasmic proteins and those that do not is being studied to learn about the mechanisms of transmembrane signaling. The main subject of this work has been the aspartate receptor, a membrane protein that directs response of *E. coli* to both maltose (when it is bound to a periplasmic binding protein) and aspartate (by direct binding). The responses to maltose and aspartate have been shown to be additive and independent. The response to one will occur whether or not the bacteria have been previously adapted to the other. The addition of both attractants simultaneously gives rise to a signal that is larger than that to either of the stimulants alone. These responses have been shown to channel eventually into the same pathways for signaling and adaptation.

Three specific arginine residues in the external portion of the receptor have been shown to be involved in binding of aspartate. Mutations at these residues affect the binding of aspartate to differing degrees, depending on the site and, to some degree, on the residue introduced. In addition, replacement of at least one of the arginines with a lysine did not impair transmission of the aspartate signal. Another of the sites could not accept lysine as a replacement. Therefore, a positively charged environment (probably a pocket) appears to be an essential, but not sufficient, determinant of binding of the negatively charged aspartate to its receptor. These mutations also had effects on the maltose response, which varied with the site altered. These observations and the earlier data on independence have led to an alternate proposal that maltose (binding protein) sends an attractant signal, because its binding site is located on the signaling pathway for aspartate, and that the three positive charges are critical to proper signal transmission, rather than directly forming an aspartate-binding site. These models are being further tested.

The aspartate receptor from *S. typhimurium* has been purified to homogeneity in detergent solution, and the laboratory is currently attempting crystallization of this protein.

III. Periplasmic Receptors of Bacterial Chemotaxis and Transport.

The periplasmic proteins are also the primary receptors for transport of the bound compounds through distinct membrane systems. Three of these proteins, the glucose/galactose, ribose, and dipeptide receptors, are being used to study the activation and recognition processes involved in chemotaxis and transport.

The ribose and glucose/galactose receptors compete for binding of a membrane chemotaxis receptor. The structure of the glucose/galactose protein from *S. typhimurium* has been solved in this laboratory to 2.4 Å resolution by the method of multiple isomorphous replacement. The correlation of the structure with the sequence similarities between these two receptor sequences has suggested regions of the protein that are probably involved in chemotactic function.

An improved method of large-scale purification of the binding proteins has been developed and has allowed purification of ribose receptor. The ribose receptors from *S. typhimurium* and *E. coli* have

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both been crystallized. The crystals of the latter are superior in quality and are currently the subject of structural analysis. The crystals show the symmetry of the orthorhombic space group $P2_12_12_1$, with the following unit cell dimensions: a = 74.59 Å, b = 88.78 Å, and c = 40.11 Å. There is a single molecule per asymmetric unit with a V_m of 2.23 Å^3/Da. Native data have been collected on a multiwire area detector to a resolution of 2.4 Å. Data (including Friedel pairs) have also been collected on a derivative crystal to 2.4 Å. Four sites of heavy metal binding have been located from Patterson and residual maps. Due to the expected similarity of the ribose-binding protein to the galactose/glucose receptor, a single derivative may prove sufficient for a structure solution, combining heavy atom phases with molecular replacement and/or solvent-flattening methods. Because the ribose receptor protein has been crystallized in the absence of bound ligand, this structure should give a picture of the more open state expected in this case.

The dipeptide-binding protein is the primary receptor for both chemotaxis toward, and transport of, dipeptides. This protein is interesting partly because of the possibility of developing suicide attractants in the form of antibiotics. In addition, the fact that a large variety of dipeptides are bound by this protein poses interesting problems concerning how the strength of ligand binding is maintained in a protein that most likely acts by closing down and burying its ligand. A method of purifying large quantities of the *E. coli* dipeptide receptor has been worked out, and this protein is currently the subject of crystallization studies. Small, apparently single, crystals have been obtained and are being studied by x-ray methods.

IV. *E. coli* Leader Peptidase.

The leader peptidase of *E. coli* has been well described as the agent by which signal sequences are removed from proteins as they are transported across membranes. Dr. Mowbray and her colleagues have developed a reliable method of large-scale purification with apparently complete inhibition of proteolytic degradation (a serious problem previously). The crystallization and successful structure solution of this protein would add valuable data about this key step of the transport event.

Dr. Mowbray is also Assistant Professor in the Departments of Biochemistry and Pharmacology at the University of Texas Southwestern Medical Center at Dallas.

STRUCTURAL STUDIES OF GENE REGULATORY PROTEINS

CARL O. PABO, PH.D., *Associate Investigator*

Dr. Pabo's research has focused on the structure and design of proteins that regulate gene expression. The laboratory is attempting to understand how proteins recognize specific sites on double-stranded DNA and how the bound proteins regulate gene expression. This information will eventually be used to design novel DNA-binding proteins for research, diagnosis, and therapy. During the past year, Dr. Pabo's research has used a high-resolution crystal structure of the λ repressor-operator complex as the basis for continued genetic and structural analysis of repressor-operator interactions. Crystallographic studies of several other DNA-binding proteins are in progress, and the laboratory has continued attempts to crystallize key regulatory proteins from the human immunodeficiency virus (HIV). The laboratory also has continued development of a database of protein-DNA interactions and development of programs for computer-aided protein design. Several tight-binding variants of the λ repressor have been designed and are being prepared for experimental tests.

I. Structural and Genetic Studies of Repressor-Operator Interactions.

Prokaryotic repressors provide useful model systems for the study of protein-DNA interactions, and Dr. Pabo's laboratory is studying the repressor from bacteriophage λ. The laboratory recently solved the crystal structure of a complex that contains the DNA-binding domain of repressor and a 20 base pair synthetic operator site. This structure showed how the helix-turn-helix motif is used for DNA recognition and led to important new insights about protein-DNA interactions. Refinement and analysis of this model have continued during the past year. Crystallographic refinement has shown how the extended amino-terminal arm fits into the major groove near the center of the operator site. Since this structural motif has not been seen in other repressor-operator complexes, site-directed mutagenesis was used to define the critical contacts. These studies show that three lysine residues in the arm play a central role in site-specific recognition.

Crystallographic studies of another prokaryotic repressor—the arc repressor from *Salmonella* bacteriophage P22—are in progress. Genetic data suggest that this protein does not use the helix-turn-helix motif for recognition, and the first heavy-atom derivative has given an initial low-resolution map of the structure. The search for other heavy-atom derivatives is in progress, and the laboratory has obtained small cocrystals of the arc repressor-operator complex.

II. Physical and Structural Studies of Eukaryotic DNA-binding Proteins.

The homeodomain is a conserved DNA-binding domain that was discovered in a set of proteins that regulate *Drosophila* development and was later observed in many other eukaryotic regulatory proteins. Although the intact proteins often are much larger, the homeodomain itself contains about 60 amino acids. Sequence comparisons and nuclear magnetic resonance (NMR) studies have indicated that the homeodomain contains a helix-turn-helix motif. Since about 100 homeodomains have been sequenced and since these bind to closely related DNA sites, the homeodomains provide an interesting system for studying protein-DNA interactions. The laboratory is attempting to crystallize representative homeodomains from yeast, *Drosophila*, and humans. Dr. Cynthia Wolberger has recently grown small cocrystals that contain the DNA-binding domain of the α2 repressor (which regulates mating type expression in yeast) and a duplex DNA fragment that contains two α2 binding sites. In collaboration with Dr. Thomas Kornberg (University of California at San Francisco), the laboratory also has grown microcrystals of the homeodomain from the *engrailed* gene of *Drosophila*.

Another structural motif—the zinc finger domain—has also been discovered in eukaryotic DNA-binding proteins. These domains each contain about 30 amino acids and have conserved cysteine and histidine residues that bind to zinc. To understand this structural motif and its role in nucleic acid recognition, Dr. Pabo and his colleagues have been studying peptides that contain zinc finger domains. The first peptide to be synthesized and characterized was a single finger that corresponded to the second domain from transcription factor IIIA of *Xenopus*. This peptide folds in the presence of zinc, but it binds DNA nonspecifically. The laboratory has now synthesized tandemly repeated domains that have higher nonspecific binding constants, and experiments are in progress to determine whether these peptides also show site-specific binding. (The

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screening procedures use the polymerase chain reaction to amplify any binding sites that occur in a pool of random synthetic fragments.) Experiments with these peptides may help determine the precise DNA-binding site for a zinc finger domain and should provide suitable molecules for cocrystallization. The tandem peptides should also be a useful starting point for the design of zinc finger proteins with novel specificities.

III. Studies of the tat and rev Proteins from HIV.

The key regulatory proteins from HIV, which is the etiological agent of AIDS, are tat and rev. Although the mechanisms of action are not fully understood, tat appears to control transcription and/or translation, while rev controls splicing and/or nuclear export of the messenger RNA. Both proteins are required for viral replication, and these are attractive targets for drug design.

The laboratory has pursued several structure and activity studies of the tat protein. In the most recent work, Dr. Alan Frankel showed that tat could be taken up by cells in tissue culture, be transported to the nucleus, and activate expression of a reporter gene. Although the biological significance of this is unclear, the study raised important questions about the mechanism of tat action and suggested new approaches for biochemical studies. Detailed structural analysis might give clues for drug design, but the laboratory has been unable to crystallize tat, and NMR studies done in collaboration with Dr. Gerhard Wagner (University of Michigan) indicate that most of purified tat protein exists as a random coil in solution. Crystallization attempts are now focusing on the rev protein (provided by Dr. Craig Rosen, Roche Institute for Molecular Biology).

IV. Computer Software for Analysis and Design of DNA-binding Proteins.

The laboratory is continuing to develop a database of protein-DNA interactions. The database includes site-specific DNA-binding proteins for which both the sequence of the protein and the sequence of the binding site are known. As more information becomes available about protein-DNA recognition, this database should be extremely useful for model-building studies and for protein design.

The laboratory also is developing strategies for computer-aided protein design. These strategies are implemented in PDB-PROTEUS, a package of programs and subroutines for computer-aided protein design. The programs allow one to test a large number of sequences or conformations when planning sequence changes or designing a new protein. Explicit criteria are used for picking the best arrangements, and these are evaluated by using detailed energy calculations and examining them on a computer graphics system. These programming strategies have recently been used to design tight-binding variants of the λ repressor. Several of these variants are being prepared for experimental tests, and this work should provide the basis for systematically designing repressors with altered specificities.

Dr. Pabo is also Associate Professor of Molecular Biology and Genetics and of Biophysics at The Johns Hopkins University School of Medicine.

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PROTEIN STRUCTURE AND FUNCTION

FLORANTE A. QUIOCHO, PH.D., *Investigator*

The three-dimensional structures of a number of proteins are being determined by x-ray crystallography in order to gain detailed molecular understanding of the functions of these proteins—especially the role of molecular recognition—and the biological processes associated with these functions.

I. Adenosine Deaminase.

Adenosine deaminase is of particular interest because impairment of its catalytic function is associated with severe combined immunodeficiency, affecting both T and B lymphocytes. Last year Dr. Quiocho reported the crystallization of two complexes of the enzyme—one with 2′-deoxycoformycin, a potent inhibitor and potential antilymphocytic compound, and the other with purine riboside, a ground-state analogue. In the past year, heavy-atom searching for phase determination of the crystal of the purine riboside complex has yielded four derivatives. Electron density maps have been calculated at 3.2 Å resolution, using phases obtained by multiple isomorphous replacement and by iterative single isomorphous replacement, which takes advantage of the crystal's high solvent content. These maps, which are similar, clearly revealed the intermolecular boundary. Interpretation of these maps is under way.

II. Antibody.

Dr. Quiocho and his colleagues have obtained crystals of Fab of a BAT123 murine monoclonal antibody raised against the gp120 coat protein of the HTLV-IIIB strain of human immunodeficiency virus. BAT123 binds to gp120 with high affinity ($K_a \sim 1.4 \times 10^{10}$ M^{-1}) at a site considered to be the most sensitive for antibody neutralization of the virus. The antibody was provided by Dr. T. W. Chang (Baylor College of Medicine).

III. Periplasmic Binding Proteins.

Binding proteins, which serve as initial receptors for bacterial active transport and chemotaxis, are ideal for detailed structure and function studies. 1) They can be easily purified in large quantities (0.2 to 2 g). 2) They are extremely stable and can be crystallized easily in excellently diffracting forms. 3) They bind diverse sets of ligands, such as carbohydrates, amino acids, and oxyanions. 4) Atomic structures of several binding proteins, in the liganded or unliganded forms, have been determined. 5) Several different binding protein genes have been cloned. The following studies have been initiated recently.

A. *Site-directed mutagenesis.* Site-directed mutagenesis provides a powerful approach to dissecting the roles of the various regions of these proteins, down to specific amino acid side chains, especially in structural integrity and biological function. With the availability of the highly refined, extremely high resolution structures of the arabinose-binding protein complexed with different sugar substrates and with the cloning of the gene coding for the protein in an *Escherichia coli* overproducer strain, the sugar-binding site region is especially suited for mutagenesis studies. This region is composed of four types of residues: type I, those that directly hydrogen bond sugar substrates; type II, those that indirectly, via bound water molecules, form hydrogen bonds with the substrates; type III, those that have nonpolar interactions with the ligand; and type IV, those that are located in the hinge between the two domains and form the base of the sugar-binding site cleft between the two domains. Dr. Vermersch has obtained the following results. 1) Substitution of Asp90, a type I residue, by Glu almost completely abolishes sugar-binding activity. 2) Replacement of type II residues, such as Gln11 by Asn and Thr147 by Gly, only diminishes sugar binding by about a half. 3) Considerable enhancement in ligand binding was achieved by substituting Met108, a type III residue, with Leu. 4) Substitution of Pro254, a type IV residue, by Gly also resulted in an enhancement of binding activity. Excellently diffracting crystals of all these mutant proteins have been obtained, paving the way for molecular understanding of the effects of these mutations.

B. *Substrate engineering.* The combination of crystallographic and binding studies for a number of deoxygenated and fluoro-substituted analogues of sugar substrates of the arabinose-binding protein is providing new insights into protein-ligand interactions. For example, D-galactose is only about twofold less tightly bound than L-arabinose, and all polar groups of both sugars interact with the pro-

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tein. On the other hand, 1-, 2-, and 6-deoxy derivatives of galactose bind 19-, 500-, and 16-fold, respectively, less tightly than galactose. Nevertheless, high-resolution structure refinements indicate no apparent difference in the structures of the complexes with galactose, 1-deoxygalactose, and 2-deoxygalactose, except for the obvious absence of corresponding hydroxyl groups on the sugars.

Refining the atomic structures of the complexes of the L-arabinose–binding protein with substrates L-arabinose, D-fucose, and D-galactose has led to the discovery that bound water molecules, coupled with localized conformational changes, can modulate substrate specificity and affinity. This suggests that there is scope for designing analogues, inhibitors, or drugs with functional groups (e.g., hydroxyl) capable of displacing bound water molecules in the ligand-binding sites of proteins and enzymes.

C. *Peptide deletions and foreign epitope insertion.* Through recombinant technology, two mutants of maltose-binding protein, each with a deletion of a different peptide segment, have been obtained. Also, the C3 neutralizing epitope for VP1 coat protein of type 1 poliovirus has been inserted into one of these deletions. All three mutant proteins have been purified and crystallized. The mutations have little effect on the transport activity of the mutant strains or on the sugar-binding activity of purified mutant proteins. Structural analysis is being undertaken to understand how the protein molecule can accommodate these large alterations and how the immunogenicity of a foreign epitope is preserved. The use of the maltose-binding protein as a vector for immunologically active peptides offers possibilities for a generation of vaccines from a nonpathogenic source.

IV. Calmodulin.

The structure of a recombinant *Drosophila melanogaster* calmodulin has been refined at 2.15 Å resolution to an R factor of 0.22.

The structure of a recombinant calmodulin missing two residues in the central helix has been determined and is being refined. Because the loss of two central helix residues leads to a loss of activity but the loss of three results in near normal activity, the orientation of the two calcium-binding domains at the ends of the helix seems to be more important for function than the helix itself.

Dr. Quiocho is also Professor of Biochemistry and of Molecular Physiology and Biophysics at Baylor College of Medicine.

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STRUCTURE AND DYNAMICS OF *DROSOPHILA* DIPLOID NUCLEI

JOHN W. SEDAT, PH.D., *Investigator*

The extremely rapid progress in the elucidation of DNA sequence information in eukaryotes has highlighted the quest for an architecture for chromosomes, from interphase to mitotic. Such an architecture will lead to a unity of structure and function. Dr. Sedat and his colleagues, in collaboration with Dr. David Agard (HHMI, University of California at San Francisco), have focused on structural questions probing nuclear and chromosomal organization. They seek answers to three questions. 1) How do interphase chromosomes fold up within the intact diploid nucleus, and how, in detail, does an interphase chromosome change as a function of progression through the cell cycle or development? 2) What is the defined interphase chromosome architecture of a specific gene? 3) Is molecular information reflected in structural attributes? Because of the extensive knowledge of its genetics, development, and biochemistry, *Drosophila melanogaster* is an ideal biological system for these integrated structural and functional studies.

The development of generalized computational-based tools for three-dimensional optical microscopy (OM) and electron microscopy (EM) of large noncrystalline subcellular structures has been described. In a resolution-overlapping fashion, data are collected with charge-coupled devices (CCD), processed to remove observational problems, and analyzed quantitatively.

The microscopy tools that are required for the structural questions are being developed, both at the OM and EM levels. The CCD for the EM430 (the HHMI state-of-the-art intermediate voltage EM) was brought on line and very high-quality data have resulted. This past year, four-dimensional OM imaging (three-dimensional images as a function of time) in living embryos was perfected and became routine. In addition, computer-controlled wavelength selection filter wheels with double-wavelength dichroic mirrors working in tandem were incorporated into the OM, so that rapid wavelength changes could be made. This allows Dr. Sedat and his colleagues to record fluorescent images from several cellular structures as a function of time. Modifications to this instrumentation, together with new software, have greatly speeded up the rate that three-dimensional data are collected. Software is being written to correct for systematic image acquisition problems, to display these data in a variety of formats, and to model and analyze (in many cases quantitatively) the intricate three-dimensional data.

Dr. Sedat and his colleagues continue to study the structure of the diploid nucleus in *Drosophila*, using the prophase and anaphase cell cycle time points as examples. Several diploid nuclear structures have been modeled, and specific chromosomes have been assigned to three-dimensional chromosomal paths. Several exciting structural features, including mirror-symmetric homologues at prophase, were described. A major effort is under way to model many examples, so that statistical comparison (one nucleus with another) can be made. Software has been written to model the chromosome paths within nuclear structures rapidly and computationally, so that large numbers of nuclear examples can be determined.

I. Real-Time Three-dimensional Optical Microscopy of Diploid Nuclear Structures.

Chromosome and nuclear structures were visualized in living embryos, using a procedure developed by Dr. Jon Minden in the laboratory of Dr. Bruce Alberts. Rhodamine-labeled histones were microinjected into embryos, where they were stably incorporated into nuclei. Three-dimensional images (e.g., 80 sets of three-dimensional data taken every 25 s) were collected. These three-dimensional images were projected into stereo pairs, followed by computational processing to remove out-of-focus information. The ability to study three-dimensional nuclear structures as a function of time in living embryos made it possible to identify functional correlations quickly. These structures showed that the four-dimensional nuclear images were excellent controls for fixation artifacts in the previous static diploid nuclear studies. Careful comparisons of the real-time and fixed-images data have indicated that the fixed samples were minimally perturbed. So far no structural discrepancy has been noted. The real-time study reveals that there were discrete chromosome condensation sites (~2–3 per nucleus) attached to the nuclear envelope that were the last to decondense during telophase. At the subsequent interphase-to-prophase transition, bright chromosome condensation spots were again observed at discrete sites on the nuclear envelope in a similar position to that of the late decondensation sites. At present it is not known if these are the identical

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chromosome sites. By following the early condensation sites at early prophase, Dr. Sedat and his colleagues could see bidirectional chromosome condensation growth from these nuclear envelope attached sites. Study of the prometaphase nuclear transition revealed that a 1.7 μm/min wave of chromosome-nuclear compaction started at the centromere end of the nucleus and proceeded toward the telomere end. These investigations are proceeding with loci identification and efforts to quantify the long-range chromosome movements observed during the nuclear cycles.

II. Structural Characterizations of a Highly Discontinuous Nuclear Lamina Localization Pattern.

What is the distribution on the nuclear surface of the major nuclear envelope protein structural lamin? Two-color, separate fluorescent labels (using a lamin-specific monoclonal antibody and a DNA-specific dye, DAPI) have been used to investigate this problem. The three-dimensional images were aligned so that comparisons were possible. The lamin distribution on the nuclear envelope was a highly discontinuous fibrillar pattern that left large voids in the nuclear periphery. This pattern did not change by varying fixation conditions or through use of polyclonal antibodies and was seen in a variety of tissue types, including mammalian cells. Careful quantitation using Fourier and real-space methods shows 0.25 μm fibers with spaces of 0.5 μm between the fibers, with approximately half of the nuclear surface occupied by the lamin structures at OM resolution. EM experiments using 10 Å gold-antibody beads and silver detection have confirmed the optical studies. Software written for this project was used to "unfold" the nuclear surface and to study the association of the lamin structures with the underlining chromosomal DNA. The vast majority of the chromosome regions that come to the nuclear surface are not associated directly with the lamin but are either significantly below the lamin structures or lie in the 0.5 μm gap between the lamin fibers. These studies are continuing.

III. Three-dimensional DNA *In Situ* Hybridization.

Until recently the structure of chromosomes was only revealed at certain times in the diploid cell cycle when chromosomes were condensed, namely prophase through telophase, or in certain specialized tissues, e.g., those containing polytenized giant chromosomes. To gain a complete understanding of the structure of diploid chromosomes during interphase (when the essential processes of gene transcription and DNA replication occur) as well as the precise relationship between specific chromosomal regions during the entire cell cycle, Dr. Sedat and his colleagues have developed new three-dimensional OM techniques.

The three-dimensional structure of the diploid nucleus is now being dissected, using high-resolution *in situ* hybridization to whole-mount *Drosophila* embryos. In this procedure, biotinylated probes homologous to 8–20 kb of single-copy chromosomal sequences are hybridized *in situ* to embryos that were previously fixed to preserve chromosome structure. The location of the hybridization probes is revealed by staining these embryos with a fluorescently tagged avidin molecule. Optical sectioning of such embryos reveals the three-dimensional location of the DNA sequence relative to chromosomal structures within the nucleus.

Preliminary results, using a limited number of hybridization probes to preblastoderm embryos, suggest that the chromosomes in diploid interphase are arranged much like those in polytene nuclei, with the centromeric regions near the embryo surface and the telomeres diametrically opposed (the Rabl orientation). Only one spot is present for each hybridization probe, indicating that the homologous loci are paired at interphase. Examination of a small number of mitotic embryos reveals an unexpected degree of ordering during cytokinesis.

Currently a wider range of hybridization probes is being used to generalize these initial observations and to determine how chromosome condensation proceeds during mitosis and how homologue pairing redevelops after the necessary structural disruptions of mitosis.

IV. Multiple-Label DNA *In Situ* Hybridization.

Last year Dr. Sedat and his colleagues completed a high-resolution molecular and structural investigation of specific polytene band/interbands. Results of experiments using these newly defined fluorescent *in situ* hybridization methods show that 1) the transcription unit of the *Notch* gene is located in the polytene band; 2) the interband contains the 5′ region of the gene, in particular the untranscribed putative regulatory sequences; 3) the interband is short (~3 kb) and extended, hence likely to have less higher-order chromosome organization.

In preparation for the application of these techniques to other genes, these laboratories have de-

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veloped a two-color, multiple fluorescent label, DNA *in situ* hybridization scheme. One DNA is labeled with a biotin probe, while the other DNA is labeled with a digoxigenin probe. Thus an internal DNA control is present during the molecular/structural alignment in polytene band/interbands. These tools are also used for *in situ* hybridization of minimally perturbed polytene chromosomes, so that three-dimensional localization questions can be asked.

Dr. Sedat is also Professor of Biochemistry and Biophysics at the University of California at San Francisco.

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CHEMICAL MECHANISMS IN CELLULAR REGULATION

PAUL B. SIGLER, M.D., PH.D., *Investigator*

Dr. Sigler's research is centered on the stereochemical mechanisms of specific macromolecular interactions involved in cellular regulation. Two main regulatory systems are emphasized: 1) the regulation of gene expression and 2) transmembrane signaling. In all cases the experimental core of the project is to determine the high-resolution crystal structure of the relevant macromolecules, both alone and in specific functional complexes. Biochemical, physicochemical, and directed mutational studies are used to test the mechanistic inferences drawn from the structural work. The long-range goal is to describe the detailed chemistry of these interactions in dynamic terms.

Regulation of gene expression will be explored in three areas, with the initial focus on the specific affinity of regulatory proteins for their DNA targets. The following systems are under study: 1) transcription in prokaryotes: *trp* repressor, *arg* repressor; 2) transcription in eukaryotes: the CCAAT/enhancer binding protein and other leucine zipper proteins, steroid receptors, GAL4, bovine papilloma virus E2, and the TATA-binding factor; 3) initiation of translation in eukaryotes: the structure and function of yeast $tRNA_i^{Met}$.

Research on transmembrane signaling is focused on the action of phospholipases that release second messengers and arachidonate, the essential biosynthetic precursor of the mediators of inflammation. The immediate aim is to establish structure-function relationships in phospholipases A_2, both alone and in complexes with their essential cofactor, Ca^{2+}, and phosphonate transition-state analogues.

I. Transcriptional Regulation: The Chemistry of Specific Protein-DNA Interactions.

A well-defined problem in transcriptional regulation is the physicochemical basis for the unusual affinity of regulatory proteins for their DNA targets. High-resolution crystallographic studies of the ligand-activated *trp* repressor have provided insight into this process and are being extended to the study of binding of the *argR* protein to tandem Arg boxes. In the past year, Dr. Sigler and his colleagues have also begun to study the interactions of a series of eukaryotic transcription factors with their DNA elements.

A. trp *repressor-operator complex.* The crystal structure of the *trp* repressor-operator complex was first solved and refined to 2.4 Å resolution and later extended to 1.9 Å with imaging-plate data from the Photon Factory (Japan's national synchrontron light source). Each of the four representations of the unique structure in the asymmetric unit shows the same specifying features in vivid detail. Since they are present in all four copies of the unique structure, they must be intrinsic to the chemistry of the interface and not an artifact of the crystal lattice. The results were startling. 1) Every hydrogen-bondable group on the surface of the repressor facing the DNA makes a hydrogen-bonded interaction with the operator, either directly or indirectly through solvent with either the phosphates or the functional groups of the major groove. There was a surprising paucity of direct interactions (only one per half repressor/operator) between protein side chains and the bases. There are at least 18 well-defined water molecules that mediate contacts to the bases and backbone. 2) Specificity apparently arises from two sources: *a*) a sequence-dependent deformation of the DNA that permits an extensive (2,900 $Å^2$) contact surface to form, presumably without significantly raising the internal energy of the DNA, and *b*) highly polarized water-mediated hydrogen bonds between the peptide N-Hs at the tip of the helix-turn-helix and the major-groove functional groups of the most mutationally sensitive base pairs of the operator.

To check the inferences drawn from the structure, Dr. Sigler and his colleagues will grow crystals in which the repressor and/or operator is changed either genetically or chemically to enhance or diminish affinity. These models should serve as a basis for developing calculations that predict sequence-specific protein-DNA affinity.

B. *Transcriptional regulation in eukaryotes.* An attempt is being made to crystallize and determine the structures of a series of eukaryotic transcription factors, both alone and in complexes with their DNA elements. Each factor is representative of a class of proteins bearing a characteristic DNA-binding motif.

In collaboration with Dr. Keith Yamamoto (University of California at San Francisco), large amounts of the DNA-binding domain of the rat glucocorticoid receptor have been expressed. This

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domain (which is characteristic of the DNA-binding domains from a large family of receptors) contains two 4S zinc fingers. Cocrystallization experiments are well under way with complexes containing the DNA target.

Dr. Steven McKnight (HHMI, The Carnegie Institution of Washington) and subsequently others have identified a family of transcription factors that dimerize through a domain called a leucine zipper (leucine heptad repeat), which has been shown to be a parallel coiled coil. Drs. Norma Duke and Jon Shuman have crystallized a complex formed by a homodimeric 20 kDa construct and its DNA target.

Recently Dr. Sigler and his colleagues purified a 62–amino acid construct derived by Tao Pan and Dr. Joseph Coleman (Yale University) from the GAL4 DNA-binding domain and expressed from an overproducing *Escherichia coli* clone. Like the glucocorticoid receptor, this DNA-binding domain contains a 4S zinc finger.

The DNA-binding domain of the E2 transactivator of bovine papilloma virus has been overexpressed from an *E. coli* clone (engineered by Steve Grossman of The University of Chicago) and purified. Efforts are under way to crystallize the DNA-binding domain both alone and in cocrystalline complexes with the E2 enhancer sequence.

The TATA sequence and its binding factor TFIID define the position and polarity of the vast majority of PolII initiation complexes. Dr. Steven Hahn (originally at the Massachusetts Institute of Technology), in collaboration with Dr. Phillip Sharp, purified and cloned TFIID from yeast. Efforts are under way to crystallize the purified protein and its TATA complex from material that Dr. Hahn has recently purified from overexpressing *E. coli* constructs.

II. Translational Control.

The crystal structure of yeast initiator tRNA has been refined to 3.0 Å with imaging-plate data from the Photon Factory. Several functionally important features emerged: A20, A59, and A60 form part of a unique and consistent sequence feature of eukaryotic initiator tRNA. They combine to make a unique hydrogen-bonded arrangement that involves the shortened backbone of the seven-membered D loop, another characteristic of this functional class. Dr. Thomas Wagner has shown that this contiguous region is the stereochemical reason why eukaryotes reject Met-$tRNA_i^{Met}$ in the peptide elongation process.

Genetic studies (Dr. U. RajBhandary, Massachusetts Institute of Technology) confirmed that the features most responsible for initiator activity are in the anticodon arm. The structure of the anticodon loop in yeast initiator tRNA is different from that of crystalline elongator tRNAs; however, the anticodon loop is involved in a crystal packing contact, and therefore one cannot be sure whether this conformational difference is intrinsic to the structure or imposed by the contact. Moreover, the anticodon arm is the most poorly ordered element in the structure, thereby obscuring the stereochemical features of the three consecutive G-C^1 base pairs at the distal end of the stem. Crystallographic and nuclear magnetic resonance (NMR) studies will be carried out on other initiator tRNAs and synthetic RNA fragments to clarify these issues further.

III. Transmembrane Signaling.

Receptor-mediated signal transduction involves the release of second messengers, either through the synthesis of cyclic nucleotides or the phospholipase-catalyzed liberation of hydrolysis products from phospholipids. These include arachidonic acid, the precursor of the eicosanoid mediators of inflammation. Inositol trisphosphate and diacylglycerol are released by phospholipase C action on phosphoinositides.

The chemistry of phospholipases is interesting, since the natural substrate is a lamellar or micellar aggregate of phospholipids rather than a soluble dispersed individual molecule. The mechanism of enzymatic attack on an aggregated substrate at the solvent-bilayer interface presents a special challenge to understanding transmembrane signaling in molecular terms.

A. *Crystallographic studies.* Dr. Sigler and his colleagues have now solved and refined a series of crotalid venom phospholipase A_2 (PLA_2) structures to 2.0 Å resolution or better. Many of the biochemical barriers to formulating a mechanism may have been leapfrogged by the crystallization of at least one and possibly more PLA_2s in complexes with a phosphonate transition-state analogue. Since one such complex is in the early stages of crystallographic refinement against 1.9 Å data, it is likely that the stereochemical mechanism for catalysis will be seen at the membrane-liquid interface.

B. *Site-directed mutagenesis.* Starting from synthetic sticky-ended oligonucleotide duplexes of 20–30 base pairs, Sandra Luisi has synthesized

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the gene for the *Crotalus atrox* enzyme, the most studied crystal structure of this laboratory. Systems for overexpressing homologous pancreatic enzymes exist and will be adapted to this protein, with a view to testing mechanistic proposals by directed mutational changes in the amino acid sequence.

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., *Assistant Investigator*

I. Three-dimensional Structure of Tumor Necrosis Factor/Cachectin.

Tumor necrosis factor (TNF) is a cytokine hormone synthesized primarily by macrophages in response to host infection by gram-negative bacteria, sepsis, or invasion by neoplastic tissue. The hormone binds to a specific 85 kDa receptor expressed on the surfaces of nearly all cells. The cytologic action of TNF follows its cellular internalization by the classic receptor-mediated endocytotic pathway. Although TNF is immediately degraded upon entry into the cell, it induces the synthesis of several nuclear oncogenes, including c-*myc* and c-*fos*. Subsequent changes in the metabolic state of cells are a function of their state of differentiation. These result in the induction of the inflammatory response, shock, and cachexia. TNF is directly cytotoxic to certain cell lines.

A structural determination of TNF was undertaken to elucidate the mechanism of receptor specificity and to search for a structural basis of its cytological activity. Results from the laboratory of Dr. Bruce A. Beutler (HHMI, University of Texas Southwestern Medical Center at Dallas) and from other laboratories suggest that receptor binding is itself not sufficient for activity, which may be potentiated by other structural determinants on the hormone.

Crystals of TNF that diffract to a resolution exceeding 2.5 Å were obtained by Michael Eck. Using a series of isomorphous heavy-metal derivatives of TNF, Eck derived an initial set of crystallographic phases. Electron density maps computed at this stage revealed that TNF forms a trimer in the asymmetric unit of the crystal, confirming reports from Dr. Sprang and other laboratories that TNF exists in solution and binds receptors as a trimeric species. Iterative cycles of symmetry averaging were used to refine the crystallographic phases, and an electron density map of sufficient quality was computed to allow the course of the polypeptide backbone to be traced and side-chain atoms to be positioned accurately. Crystallographic refinement assisted by molecular dynamics "simulated annealing" [using the program package XPLOR by Dr. Axel T. Brünger (HHMI, Yale University)] reduced the crystallographic R value to 0.23.

Subunits of TNF, which are composed entirely of antiparallel β-sheet structure, bear a striking topological resemblance to coat proteins of icosahedral RNA plant and animal viruses. Subunits share an extensive interface about the noncrystallographic threefold axis of symmetry, in an interaction stabilized by both polar and hydrophobic contacts. A putative receptor-binding site was identified by locating, in the three-dimensional atomic model, residues that are identically conserved between TNF and the related lymphokine, lymphotoxin, which also binds to the TNF receptor. This analysis revealed that lymphotoxin is probably a trimer and that the receptor-binding site in both molecules is localized to a patch of residues at the base of the trimer, near the amino and carboxyl termini of the subunits.

Further studies of TNF mutants with altered receptor binding and cytological activities are planned, in collaboration with Dr. Beutler, to test the present model for the binding site and to delineate features of the binding site by directed mutagenesis. A second, long-range goal is to identify the molecular determinants of the hormone transduction events subsequent to receptor binding.

II. Structural Studies of Annexins.

The annexins comprise a large family of calcium-binding proteins associated, in a variety of cell types, with the cytoskeleton or with microsomal membranes. These proteins, which include the calelectrins, endonexins, lipocortins, and calbindins, appear to have evolved by serial duplication of an exon encoding a 70- to 90-residue, possibly α-helical, domain. Members of the annexin family with molecular weights in the 32–36 kDa range comprise four such repeats, while the 67K proteins appear to be duplications of the 32–36K protein domains. Both molecular weight classes demonstrate a phospholipid-dependent calcium-binding activity, and several of the annexins are phosphorylated at serine or tyrosine residues. The activity or function of none of these proteins has been identified, but it is possible that certain members of the family are involved in potentiating membrane fusion in a calcium-regulated manner.

Using preparative methods developed by Drs. Thomas H. Südhof (HHMI) and Gary Reynolds (University of Texas Southwestern Medical Center at Dallas), the laboratory has purified both the 32K lipocortin III and the 67K calelectrin to homogeneity. Crystals of both proteins have been grown in

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the presence of calcium. Lipocortin III crystals, belonging to the rhombohedral space group R3 with cell constants a = 121.3 Å and c = 83.0 Å (hexagonal setting), diffract beyond 2.5 Å resolution. It is expected that the determination of these structures will reveal a novel calcium-binding motif; future structural studies of these proteins in the presence and absence of calcium and phospholipid components should elucidate the mechanism of cooperativity between phospholipid and calcium binding.

III. Allosteric Regulation of Glycogen Phosphorylase.

The laboratory continues its exploration of the structural mechanism of homotropic and heterotropic cooperativity in this enzymatic regulator of glycogen metabolism. Previous studies, in collaboration with Dr. Elizabeth Goldsmith (University of Texas Southwestern Medical Center at Dallas) and Dr. Robert Fletterick (University of California at San Francisco), have elucidated the structural basis for phosphoregulation and the nature of the structural changes that accompany cooperative substrate binding. Recent work has focused on the description of the conformational changes caused by the simultaneous presence of substrate and two activators, adenosine monophosphate and oligosaccharides. This work has shown that the crystalline enzyme is able to catalyze the degradation of oligosaccharides to glucose-1-phosphate, leaving a product complex bound to the active site. The extensive conformational changes that result from this reaction are presently being analyzed. Crystallographic studies are also in progress to identify the binding site of divalent and trivalent ions, including magnesium and calcium, both of which promote phosphorylase activation.

IV. Structural Studies of G Regulatory Proteins.

Efficient *Escherichia coli* expression systems have been successfully exploited by Dr. Alfred Gilman (University of Texas Southwestern Medical Center at Dallas) to yield milligram quantities of the β-adrenergic receptor-responsive G protein regulator of adenylate cyclase, Gsα. In collaboration with Dr. Gilman's laboratory, crystallization trials (in the presence of nonhydrolyzable guanosine triphosphate analogues) of Gsα and active tryptic fragments of Gsα are now under way. The three-dimensional structures of these molecules should contribute to the understanding of the intricate mechanism of transmembrane signal transduction utilized by this ubiquitous family of regulatory proteins.

Dr. Sprang is also Assistant 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*

Single-crystal x-ray crystallography combined with molecular genetics provides powerful tools for understanding the relationships between the structure of a macromolecule and its biological function. Dr. Steitz has been using these tools to elucidate the structure and function of proteins and nucleic acids, particularly those that are involved in DNA replication, DNA recombination, the regulation of transcription, and some aspects of protein synthesis. The major achievements of the past year include the determination of the structure of $tRNA^{Gln}$-synthetase complex and significant progress toward the structure determination of the *Escherichia coli rec*A, CAP-DNA complex, and Klenow fragment–DNA complex.

I. Protein-DNA Interactions.

The catabolite gene activator protein (CAP) from *E. coli* is a dimer of 22,500-molecular-weight subunits that activates transcription from certain *E. coli* operons in the presence of cAMP. This protein is the first of the sequence-specific DNA-binding proteins whose crystal structure was determined; each subunit was found to consist of a domain that binds cAMP and a smaller domain involved in binding the DNA.

X-ray data at 3.0 Å resolution from CAP cocrystallized with a 31 bp DNA fragment confirm the overall aspects of the CAP-DNA model built earlier using electrostatic calculations and other considerations. The positions of BrdU residues at each end of this DNA are close to those predicted from the model and show that the DNA is sharply bent around CAP, with a total bend angle of ~140°. Complete determination of this crystal structure is in progress.

Resolvase is a site-specific recombination protein from the transposable element gamma-delta. A few years ago Dr. Steitz and Dr. Nigel Grindley demonstrated that this protein contains a 43–amino acid carboxyl-terminal domain that binds to DNA in a sequence-specific fashion and a 140–amino acid amino-terminal domain that forms the oligomeric interactions and catalyzes the recombination reaction. Large, single crystals were grown of both the amino-terminal catalytic domain and the intact protein that diffract to ~2.4 and 3.5 Å resolution, respectively.

Two mutant proteins that each contain a cysteine residue introduced to provide heavy-atom–binding sites were used to solve the structure of the catalytic domain. This immediately provided an understanding of mutations that abolish recombination without affecting DNA binding. Cocrystals of the intact protein with a 30 bp DNA fragment have been obtained, and attempts to cocrystallize with the 120 bp *res* site are in progress.

*E. coli rec*A protein plays a major and essential role in general recombination. Although crystals of *rec*A were grown 10 years ago, a high-resolution electron density map showing features of the protein was only achieved in the past six months. One additional heavy-atom derivative will be required to solve this structure completely.

The large proteolytic fragment (Klenow fragment) of DNA polymerase I of *E. coli* was crystallized and its structure determined in Dr. Steitz's laboratory several years ago. This 68,000-molecular-weight protein catalyzes both the DNA polymerase and a 3′–5′ exonuclease reaction. Dr. Steitz and his colleagues have proven that the polymerase active site resides on the larger carboxyl-terminal domain and the exonuclease active site resides on the smaller amino-terminal domain.

Mutant Klenow fragment proteins have been made in collaboration with Dr. Grindley and Dr. Catherine M. Joyce. A change of Asp-424 to Ala or of both Glu-355 and Asp-357 to Ala produces Klenow fragment proteins that retain full polymerase activity but exhibit no exonuclease activity. High-resolution crystal structure analyses of these proteins show that the mutations produce no detectable change in the protein structure but alter the binding of essential metal ions at the exonuclease active site. Structural and functional studies of other site-directed mutant proteins altered in both the polymerase and exonuclease active sites are being pursued.

Two cocrystal forms of Klenow fragment complexed with DNA have been grown—one with DNA bound at the exonuclease active site and one with DNA at the polymerase active site. The structure of the editing complex shows that the enzyme has denatured the duplex DNA and bound four single-stranded nucleotides to the 3′–5′ exonuclease active site. An electron density map of the DNA complex at the polymerase active site shows a repositioning of protein around the DNA-binding cleft, including a portion that is dis-

Continued

ordered in the absence of DNA. Electron density for the DNA has not yet been interpreted unambiguously.

II. Protein-RNA Interaction.

The crystal structure of *E. coli* glutaminyl-tRNA synthetase (GlnRS) complexed with its cognate $tRNA^{Gln}$ and ATP has been solved at 2.8 Å resolution. The enzyme consists of four domains arranged to give an elongated molecule with an axial ratio >3 to 1. Its interactions with the tRNA extend from the anticodon to the acceptor stem along the entire inside of the "L" of the tRNA. The complexed tRNA retains the overall conformation of the yeast $tRNA^{Phe}$, with two major differences: the 3′ acceptor strand of $tRNA^{Gln}$ hairpins back toward the inside of the "L" with the disruption of the final base pair of the acceptor stem, and the anticodon loop adopts a conformation not seen in any of the previously determined tRNA structures. Specific recognition elements identified so far involve 1) enzyme contacts with the two-amino groups of guanine via the tRNA minor groove in the acceptor stem at G2 and G3, 2) interactions between the enzyme and the anticodon nucleotides, and 3) the ability of the nucleotides G_{73} and U_1-A_{72} of the cognate tRNA to assume a conformation stabilized by the protein at a lower free-energy cost than noncognate sequences.

The central domain of this synthetase binds ATP, glutamine, and the acceptor end of the tRNA as well as making specific interactions with the acceptor stem. It exhibits a strong structural similarity to the dinucleotide-binding motifs of the tyrosyl- and methionyl-tRNA synthetases, suggesting that all synthetases may have evolved from a common domain of this type capable of recognizing the acceptor stem of the cognate tRNA.

III. Human Immunodeficiency Virus (HIV) Proteins.

HIV reverse transcriptase has been purified from an *E. coli* expression system. Specific tRNA primed initiation from RNA and DNA templates is being studied to define a system suitable for cocrystallization of protein complexed with tRNA and an appropriate template.

HIV Tat protein has been expressed as a fusion with the cAMP-binding domain of CAP. Both the purified chimera and the cleaved Tat bind TAR (transactivation responsive region) RNA quantitatively and specifically. Having now established that Tat may function by binding to TAR RNA, Dr. Steitz and his colleagues will cocrystallize the protein-RNA complex.

IV. Other Enzymes.

Work on the structure of yeast hexokinase in Dr. Steitz's laboratory in the 1970s showed that the binding of glucose produced a large and essential conformational change. Study of the ternary complex of this enzyme with glucose and ATP is now being undertaken to examine the details of catalysis in this enzyme.

Dr. Steitz is also Professor of Molecular Biophysics and Biochemistry and of Chemistry at Yale University.

PUBLICATIONS

Books and Chapters of Books

Beese, L.S., and Steitz, T.A. 1989. Structure of *E. coli* DNA polymerase I, large fragment, and its functional implications. In *Nucleic Acids and Molecular Biology* (Eckstein, F., and Lilley, D., Eds.). Heidelberg: Springer-Verlag, pp 28–43.

Articles

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STRUCTURAL MOLECULAR BIOLOGY

DON C. WILEY, PH.D., *Investigator*

Dr. Wiley's laboratory has determined the structures of membrane glycoproteins in order to study the mechanism of their biological activities. Currently the structures of three polymorphic proteins—the influenza virus hemagglutinin (HA), the human class I histocompatibility antigens HLA-A2 and HLA-A28, and the variable-surface glycoprotein from trypanosomes—are being studied by high-resolution x-ray crystallography. In each case, biochemical, physical, chemical, and recombinant DNA methods are being used to study the function of the proteins.

I. Influenza Virus.

The influenza hemagglutinin glycoprotein has at least three activities during viral infection. 1) It binds the virus to a cell surface by recognizing a sialic acid receptor. 2) It accomplishes virus-cell entry by fusing the virus membrane to the cell's membrane after being triggered by the low pH of an endocytic vesicle. 3) It undergoes structural changes from year to year that result in antigenic variation and the reoccurrence of influenza epidemics in previously infected populations.

A. *Receptor binding.* The structures of the HA from the 1968 Hong Kong virus and two single–amino acid substitution mutants were determined complexed with trisaccharide analogues of the cellular receptors of the virus. Those structures have now been refined, and a molecular model for the recognition of sialic acid by the virus has been published. The laboratory has begun synthesizing sialoside derivatives with substituents at various positions on the saccharide ring, both to permit further x-ray analyses to confirm the current model by locating specific positions on the ring and to explore which positions will accept substituents and still fit into the HA-binding site. A 4-0 acetylated sialoside has been complexed with crystalline HA, and x-ray data are being collected.

A nuclear magnetic resonance (NMR) binding assay developed in collaboration with Dr. George Whitesides (Harvard University) has been used to determine the equilibrium dissociation constant between hemagglutinin from wild-type and mutant viruses with α(2,6)- and α(2,3)-linked sialosides. The dissociation constants are weak, ~2 mM. They are the same for monosaccharides and trisaccharides, supporting the crystal structure data, which indicate that the HA interacted strongly with only the sialic acid in a trisaccharide.

A large NMR chemical shift observed for the sialic acid *N*-acetyl methyl group is also consistent with the location of that methyl group over a tryptophan ring, as observed in the crystal structure. Such a ring amount shift would be expected from the crystal structure, arguing that the crystal structure of the complex is the same as the solution structure reported by the NMR signal.

Efforts to design molecules to block receptor binding are beginning.

B. *Membrane fusion.* A series of single-site mutations has been constructed on a cloned gene of the HA to explore the membrane fusion activity of the HA. Mutations that destabilize the trimer interaction in the globular HA_1 chain raise the pH optimum for membrane fusion, arguing that the domains must rearrange during membrane fusion. Disulfide bonds introduced to lock the trimer interfaces covalently with the HA_1 domains destroy fusion activity, while retaining receptor binding and antibody recognition. This indicates that these domains must be able to rearrange for fusion to occur. The experiments suggest that if the trimer interface could be stabilized by a drug molecule, membrane fusion, and therefore virus infectivity, could be prevented.

The structure of one of the single-site substitutions, Asp-112 HA_2 to Gly mutant HAs with an increased pH optimum for membrane fusion, has been determined by x-ray diffraction. It shows a loss of four intramolecular hydrogen bonds that stabilize the location of the amino-terminal "fusion peptide" of the HA, suggesting the origin of the mutant phenotype.

II. Human Histocompatibility Antigens.

The structure of HLA-A2, a class I histocompatibility antigen, has been refined to 2.7 Å resolution. The refined structure reveals details of the putative antigenic peptide found in a prominent cleft on the molecular surface. A series of well-defined pockets that appear to be recognition sites for amino acid side chains of processed antigens are now visible in the cleft.

A second human histocompatibility antigen, HLA-

Continued

A28, which has changes at 13 of the 16 most polymorphic amino acid positions, has been determined and refined to 2.6 Å resolution. Because of its extensive polymorphism, A28 would appear to be a good model for the effect of polymorphism on class I molecules. The structure of A28 is similar to A2. The prominent cleft is similar and also contains extra density, presumably the image of a peptide or collection of peptides. The polymorphic substitutions result in significant changes in the character of the binding cleft. One particular region that is relatively flat in HLA-A2 has a deep pocket lined with two unneutralized carboxylate groups, suggesting a role in binding a positively charged portion of a foreign peptide. A significant peak of the extra density is found in this pocket.

Crystals of the human class II histocompatibility antigen HLA-DR1 have been grown in collaboration with Dr. Jack Strominger (Harvard University). It appears possible to add peptides to these crystals, which have been shown in the laboratory to bind to HLA-DR1.

Efforts to bind peptides to HLA-A2 are also in progress.

III. Variable-Surface Glycoprotein.

The structures of two variable-surface glycoproteins from the membrane of the parasite *Trypanosoma brucei* have been determined to 2.8 Å resolution. The molecules lack statistically significant sequence homology, yet have extremely similar structures.

IV. Human Immunodeficiency Virus (HIV).

In collaboration with Dr. Stephen C. Harrison (HHMI, Harvard University), Dr. Wiley has initiated studies on proteins from HIV and its cellular receptor. A number of crystal forms of CD4 have been obtained.

Dr. Wiley is also Professor of Biochemistry and Biophysics at Harvard University.

PUBLICATIONS

Articles

Metcalf, P., Down, J.A., Turner, M.J., and Wiley, D.C. 1988. Crystallization of amino-terminal domains and domain fragments of variant surface glycoproteins from *Trypanosoma brucei brucei*. *J Biol Chem* 263:17030–17033.

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Wharton, S.A., Martin, S.R., Ruigrok, R.W.H., Skehel, J.J., and Wiley, D.C. 1988. Membrane fusion by peptide analogues of influenza virus haemagglutinin. *J Gen Virol* 69:1847–1857.

HHMI-NIH RESEARCH SCHOLARS AT THE NATIONAL INSTITUTES OF HEALTH

The Howard Hughes Medical Institute–National Institutes of Health Research Scholars Program for medical students was initiated in the summer of 1985. Students at medical schools in the United States apply for the program in the autumn of the preceding year; after initial review by HHMI-NIH Program Committee members, about two-thirds are brought for interview to the NIH in March. Those selected are invited to join the program. After acceptance the students select an area of research interest from the five HHMI programs: Cell Biology and Regulation, Genetics, Immunology, Neuroscience, or Structural Biology. Prior to arrival in Bethesda, the student is assigned to a Senior Scientist of the NIH in the scholar's area of interest, who serves as an advisor. Each student begins the academic year by interviewing with a number of NIH scientists, from which the student selects a research preceptor for the ensuing year. In the summer of 1989, 34 students from 23 medical schools began their research. They were joined by 11 students from the previous group, who are continuing for a second year of work on the recommendation of their preceptors.

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

HHMI-NIH MEDICAL STUDENTS 1985–1986

Scholar	*School*	*Advisor*	*Program*	*Preceptor*
Peter W. Abcarian	Dartmouth Medical School	Daniel Camerini-Otero, M.D., Ph.D.	Molecular Genetics	Howard A. Nash, M.D., Ph.D.
Anthony A. Asher	Wayne State University	Warner C. Greene, M.D., Ph.D.	Immunology	Steven A. Rosenberg, M.D., Ph.D.
Andrew J. Boyd	University of New Mexico	Phillip G. Nelson, M.D., Ph.D.	Neuroscience	Agu Pert, Ph.D.
Jodell J. Boyle	Duke University	Claude B. Klee, M.D.	Metabolic Control	Joram Piatigorsky, Ph.D.
Douglas P. Clark	University of Illinois	George Khoury, M.D.	Molecular Genetics	Michael M. Gottesman, M.D.
Brock L. Eide	University of Washington	Bruce D. Weintraub, M.D.	Metabolic Control	Allen M. Spiegel, M.D.
Gregory M. Fox	University of Michigan	William Paul, M.D.	Immunology	Igal B. Gery, Ph.D.
Gary S. Gottesman	University of Michigan	Arthur W. Nienhuis, M.D.	Molecular Genetics	Michael A. Zasloff, M.D., Ph.D.
Hratchia Havoudjian	University of North Carolina	Richard D. Klausner, M.D.	Metabolic Control	Phillip Skolnick, Ph.D.
Victor Ho	Duke University	Phillip G. Nelson, M.D., Ph.D.	Neuroscience	Louis Sokoloff, M.D.
Norman Hogikyan	University of Michigan	Roscoe O. Brady, M.D.	Neuroscience	Rachael Myerowitz, Ph.D.
Alan S. Krasner	Northwestern University	Claude B. Klee, M.D.	Metabolic Control	Igor B. Dawid, Ph.D.
Janet E. Lewis	Medical College of Ohio	Warner C. Greene, M.D., Ph.D.	Immunology	Jeffrey Bluestone, Ph.D.
Richard D. Lopez	Stanford University	William Paul, M.D.	Immunology	David Margulies, M.D., Ph.D.
Bobak Mozayeni	Albany Medical College of Union University	Ronald H. Schwartz, Ph.D.	Immunology	John N. Weinstein, M.D., Ph.D.
Michael W. Myers	University of Michigan	Roscoe O. Brady, M.D.	Neuroscience	Robert A. Lazzarini, Ph.D.
Leslie A. Parent	Duke University	Warner C. Greene, M.D., Ph.D.	Immunology	Dinah S. Singer, Ph.D.
Maitray D. Patel	University of Michigan	William Paul, M.D.	Immunology	Richard D. Klausner, M.D.
Caroline C. Philpott	Duke University	Richard D. Klausner, M.D.	Metabolic Control	Jurrien Dean, M.D.
Edwin P. Rock	University of Pittsburgh	Daniel Camerini-Otero, M.D., Ph.D.	Molecular Genetics	Russell J. Howard, Ph.D.
Michael Schneck	Cornell University	R.O. Brady, M.D. and P.G. Nelson, M.D., Ph.D.	Neuroscience	Dale E. McFarlin, M.D.
Virginia Stark-Vancs	University of Oklahoma	George Khoury, M.D.	Molecular Genetics	W. French Anderson, M.D.
Mary Jo Viglione	University of Pennsylvania	Arthur W. Nienhuis, M.D.	Molecular Genetics	Jeffrey Schlom, Ph.D.

Continued

HHMI-NIH MEDICAL STUDENTS 1986–1987

Scholar	*School*	*Advisor*	*Program*	*Preceptor*
Katrin Andreasson	Columbia University College of Physicians and Surgeons	Philip G. Nelson, M.D., Ph.D.	Neuroscience	Pen Loh, Ph.D.
Eddy Anglade	Yale University	William Paul, M.D.	Immunology	Richard J. Hodes, M.D.
*Douglas Clark	University of Illinois, Chicago	George Khoury, M.D.	Molecular Genetics	Michael M. Gottesman, M.D.
Joseph Cohen	Albany Medical College of Union University	Ronald H. Schwartz, Ph.D.	Immunology	Ronald N. Germain, M.D., Ph.D.
Bruce Conklin	Case Western Reserve University	Phillip G. Nelson, M.D., Ph.D.	Neuroscience	Julius Axelrod, Ph.D.
David Coran	University of Michigan	Thomas A. Waldmann, M.D.	Immunology	David I. Cohen, M.D.
Christopher Corsico	Pennsylvania State University	George Khoury, M.D.	Molecular Genetics	Bruce H. Howard, M.D.
Randall Cron	University of California, Riverside	William Paul, M.D.	Immunology	Jeffrey Bluestone, Ph.D.
Douglas Feltner	University of Michigan	Arthur W. Nienhuis, M.D.	Molecular Genetics	Mark Israel, M.D.
David Gudeman	University of Kansas	Philip G. Nelson, M.D., Ph.D.	Neuroscience	Carl R. Merrill, M.D.
Helen Hollingsworth	New York University	Daniel Camerini-Otero, M.D., Ph.D.	Molecular Genetics	Nancy C. Nossal, Ph.D.
Paul Hsieh	University of Michigan	Claude B. Klee, M.D.	Metabolic Control	Maurice B. Burg, M.D.
Burt Hutto	Medical University of South Carolina	Roscoe O. Brady, M.D.	Neuroscience	Philip W. Gold, M.D.
Laura Ibsen	University of California	Michael Brownstein, M.D., Ph.D.	Neuroscience	Charles R. Gerfen, Ph.D.
Eric Kaldjian	University of Michigan	Richard D. Klausner, M.D.	Immunology	Alfred Singer, M.D.
Vera Kowal	University of Michigan	George Khoury, M.D.	Molecular Genetics	Dean H. Hamer, Ph.D.
Christine Lee	Duke University	David H. Sachs, M.D.	Immunology	Eric O. Long, Ph.D.
Joon Lee	Duke University	Ronald H. Schwartz, Ph.D.	Immunology	H.D. (Sandy) Morse, M.D.
Susan Lontkowski	Duke University	Gary Felsenfeld, Ph.D.	Molecular Genetics	Rachael Myerowitz, Ph.D.
*Richard D. Lopez	Stanford University	William Paul, M.D.	Immunology	David Margulies, M.D., Ph.D.
Alice Ma	University of Michigan	Steven P. Wise, Ph.D.	Neuroscience	Carole L. Jelsema, Ph.D.
Michael Measom	University of Utah	Michael Brownstein, M.D., Ph.D.	Neurosience	Ray Johnson, Ph.D.
Elias Najem	New Jersey Medical School	Michael Brownstein, M.D., Ph.D.	Neuroscience	George H. Yoakum, Ph.D.
David Polomis	University of Michigan	Bruce D. Weintraub, M.D.	Metabolic Control	Jesse Roth, M.D.
David Pombo	University of Utah	Daniel Camerini-Otero, M.D., Ph.D.	Molecular Genetics	Michael F. Good, M.D.
Christopher Rall	Medical College of Virginia, VCU	Jack Orloff, M.D.	Metabolic Control	Bryan Brewer, M.D.
Paula Ross	University of Michigan	Claude B. Klee, M.D.	Metabolic Control	Richard D. Klausner, M.D.
Photini Sinnis	Dartmouth Medical School	Arthur W. Nienhuis, M.D.	Molecular Genetics	Thomas Wellens, M.D., Ph.D.
Jeffrey Sussman	University of California, Los Angeles	William Paul, M.D.	Immunology	Jonathan D. Ashwell, M.D.
Marie Tani	Case Western Reserve University	Steven Wise, Ph.D.	Neuroscience	Robert A. Lazzarini, Ph.D.
Charles Tsai	University of Michigan	Gary Felsenfeld, Ph.D.	Molecular Genetics	Carl Wu, Ph.D.
Philip Wang	Harvard Medical School	Michael Brownstein, M.D., Ph.D.	Neuroscience	Dennis L. Murphy, M.D.
Louis Weimer	Emory University	Steven Wise, Ph.D.	Neuroscience	Clarence J. Gibbs, Ph.D.
Rosalind Welty	University of Michigan	Arthur Nienhuis, M.D.	Molecular Genetics	Daniel W. Nebert, M.D.
Steven Wolff	Duke University	Richard D. Klausner, M.D.	Immunology	Robert S. Balaban, Ph.D.
Michael Wood	University of Michigan	David Sachs, M.D.	Immunology	R. Michael Blaese, M.D.
Suzanne Zorn	Pennsylvania State University	Thomas A. Waldmann, M.D.	Immunology	Ajay Bakhshi, M.D.

*Second year

Continued

HHMI-NIH MEDICAL STUDENTS 1987–1988

Scholar	*School*	*Advisor*	*Program*	*Preceptor*
Matthew Anderson	University of Miami	Michael Brownstein, M.D., Ph.D.	Neuroscience	Izja Lederhendler, Ph.D.
Jeffrey Balke	University of Minnesota	William Paul, M.D.	Immunology	Uli Siebenlist, Ph.D.
Laurie Beitz	University of North Carolina	Jack Orloff, M.D.	Cell Biology and Regulation	Simeon Taylor, M.D., Ph.D.
Paul Bohjanen	University of Michigan	William Paul, M.D.	Immunology	Richard Hodes, M.D.
David Bradley	University of Minnesota	Michael Brownstein, M.D., Ph.D.	Neuroscience	Larry Weinberger, Ph.D.
Alex Cech	Duke University	Gilbert Ashwell, M.D.	Cell Biology and Regulation	Stuart A. Aaronson, M.D
Catherine Chen	Harvard Medical School	Henry Metzger, M.D.	Immunology	Richard Klausner, M.D.
Kenneth Colina	Baylor College of Medicine	Joram Piatigorsky, Ph.D.	Immunology	Eric A. Ottesen, M.D.
*Bruce Conklin	Case Western Reserve University	Phillip G. Nelson, M.D., Ph.D.	Neuroscience	Julius Axelrod, Ph.D.
*Christopher Corsico	Pennsylvania State University	George Khoury, M.D.	Molecular Genetics	Bruce Howard, M.D.
*Randall Cron	University of California, Riverside	William Paul, M.D.	Immunology	Jeffrey Bluestone, Ph.D
John Eng	University of Wisconsin	Claude Klee, M.D.	Cell Biology and Regulation	Robert Balaban, Ph.D.
Ahron Friedberg	SUNY at Buffalo	Flossie Wong-Staal, Ph.D.	Molecular Genetics	W. French Anderson, M.D.
Alicia Fry	University of Cincinnati	Thomas Waldmann, M.D.	Immunology	Louis Matis, M.D.
James Harbour	Johns Hopkins University	Michael Brownstein, M.D., Ph.D.	Molecular Genetics	John D. Minna, M.D.
Mark Holm	University of Minnesota	Joram Piatigorsky, Ph.D.	Molecular Genetics	Gary Felsenfeld, Ph.D.
Eric Hsi	University of Michigan	Henry Metzger, M.D.	Immunology	Lawrence Samelson, M.D.
James Huang	Baylor College of Medicine	Bruce Howard, M.D.	Molecular Genetics	Robert A. Lazzarini, Ph.D.
Eric Janis	Johns Hopkins University	Henry Metzger, M.D.	Immunology	Ronald Schwartz, Ph.D.
Velissarios Karacostas	Eastern Virginia Medical School	Flossie Wong-Staal, Ph.D.	Molecular Genetics	Bernard Moss, M.D., Ph.D.
Judy Kim	Johns Hopkins University	Gilbert Ashwell, M.D.	Cell Biology and Regulation	Jay Berzofsky, M.D., Ph.D.
Jody Krosnick	Temple University	William Paul, M.D.	Immunology	Steven Rosenberg, M.D., Ph.D.
Thomas Lietman	Columbia University	Michael Brownstein, M.D., Ph.D.	Molecular Genetics	Graeme Wistow, Ph.D.
Steve Maxfield	Duke University	Thomas Waldmann, M.D.	Immunology	Ethan M. Shevach, M.D.
Joseph Moates	University of Alabama	Gilbert Ashwell, M.D.	Cell Biology and Regulation	Fred Wondisford, M.D.
Lewis Morgenstern	University of Michigan	Phillip Nelson, M.D., Ph.D.	Neuroscience	Edward Ginns, M.D., Ph.D.
Laura Napolitano	University of Rochester	Thomas Waldmann, M.D.	Immunology	Gilbert Jay, Ph.D.
David Pezen	Loyola-Stritch School of Medicine	Roscoe Brady, M.D.	Neuroscience	Malcolm Martin, M.D
Michael Rees	University of Michigan	Henry Metzger, M.D.	Immunology	Alfred Singer, M.D.
Mark Sands	Northwestern University	Bruce Howard, M.D.	Molecular Genetics	Michael B. Sporn, M.D.
Andrew Scharenberg	University of Michigan	Phillip Nelson, M.D., Ph.D.	Neuroscience	Daniel Alkon, M.D.
Stephen Skirboll	University of Pennsylvania	Roscoe Brady, M.D.	Neuroscience	Irwin Kopin, M.D.
Spencer Smith	Duke University	Arthur Nienhuis, M.D.	Molecular Genetics	Michael M. Gottesman, M.D.
Gregory Springett	Harvard Medical School	Arthur Nienhuis, M.D.	Molecular Genetics	W. French Anderson, M.D.
James Teener	University of Michigan	Roscoe Brady, M.D.	Neuroscience	Dmitry Goldgaber, Ph.D.
Michelle Verplank	Michigan State University	Phillip Nelson, M.D., Ph.D.	Neuroscience	
John Viola	Jefferson Medical College	Roscoe Brady, M.D.	Neuroscience	Louis Sokoloff, M.D.
*Steven Wolff	Duke University	Richard D. Klausner, M.D.	Cell Biology and Regulation	Robert Balaban, Ph.D.

*Second year

Continued

HHMI-NIH MEDICAL STUDENTS 1988–1989

Scholar	*School*	*Advisor*	*Program*	*Preceptor*
Rhoda Alani	University of Michigan	Bruce Howard, M.D.	Molecular Genetics	Michael J. Birrer, M.D., Ph.D.
Kirsi Allison	Dartmouth Medical School	Thomas Waldmann, M.D.	Immunology	Warren Strober, M.D.
*Matthew Anderson	University of Miami	Michael Brownstein, M.D., Ph.D.	Neuroscience	Daniel Alkon, M.D.
Sarah Barksdale	University of California (UCSF)	Bruce Howard, M.D.	Molecular Genetics	John D. Minna, M.D.
Douglas Bell	Harvard Medical School	Roscoe Brady, M.D.	Neuroscience	Meg Palmatier, Ph.D.
Kevin Black	Duke University	Roscoe Brady, M.D.	Neuroscience	Edward I. Ginns, M.D., Ph.D.
*Paul Bohjanen	University of Michigan	William Paul, M.D.	Immunology	Richard Hodes, M.D.
*David Bradley	University of Minnesota	Michael Brownstein, M.D., Ph.D.	Neuroscience	Cary Weinberger, Ph.D.
Stephen Boorstein	University of Michigan	Thomas Waldmann, M.D.	Immunology	Richard E. Clark, M.D.
Donna Brezinski	Yale University	Bruce Howard, M.D.	Molecular Genetics	Stuart A. Aaronson, M.D.
Lourdes De Armas	University of Puerto Rico	Thomas Waldmann, M.D.	Immunology	Stephen Straus, M.D.
Richard Ellis	Southern Illinois University	Rachel Myerowitz, Ph.D.	Molecular Genetics	Nancy Nossal, Ph.D.
*John Eng	University of Wisconsin	Claude Klee, M.D.	Cell Biology and Regulation	Robert Balaban, Ph.D.
Elizabeth Farr	University of Michigan	Thomas Waldmann, M.D.	Immunology	Bruce Weintraub, M.D.
David Frucht	Duke University	Henry Metzger, M.D.	Immunology	John Leonard, M.D.
*Alicia Fry	University of Cincinnati	Thomas Waldmann, M.D.	Immunology	Louis Matis, M.D.
William Greene	University of California (UCSF)	Roscoe Brady, M.D.	Neuroscience	Ward Odenwald, Ph.D.
John Hegarty	Pennsylvania State University	Thomas Kindt, Ph.D.	Immunology	Scott Koenig, M.D.
Carol Ann Huff	Baylor College of Medicine	Rachel Myerowitz, Ph.D.	Molecular Genetics	
Robert Kanterman	University of Miami	Gilbert Ashwell, M.D.	Cell Biology and Regulation	Julius Axelrod, Ph.D.
*Velissarios Karacostas	Eastern Virginia Medical School	Flossie Wong-Staal, Ph.D.	Molecular Genetics	Bernard Moss, M.D., Ph.D.
Robert Lechleider	University of Illinois, Chicago	Richard D. Klausner, M.D.	Cell Biology and Regulation	Michael Sporn, M.D.
Mitchell Lee	East Carolina University	Gilbert Ashwell, M.D.	Cell Biology and Regulation	John Hanover, Ph.D.
*Thomas Lietman	Columbia University	Michael Brownstein, M.D., Ph.D.	Molecular Genetics	Graeme Wistow, Ph.D.
Abhijit Limaye	University of Washington	Phillip Nelson, M.D., Ph.D.	Neuroscience	Eric Ottesen, M.D.
Bertha Lin	University of Michigan	Joram Piatigorsky, Ph.D.	Molecular Genetics	Warren J. Leonard, M.D.
William Lipham	Baylor College of Medicine	Arthur Nienhuis, M.D.	Cell Biology and Regulation	Igal Gery, Ph.D.
Josh McDonald	Duke University	Joram Piatigorsky, Ph.D.	Molecular Genetics	Curtis Harris, M.D.
Mark Miller	Yale University	Al Rabson, M.D.	Immunology	D. Carleton Gajdusek, M.D.
*Laura Napolitano	University of Rochester	Thomas Waldmann, M.D.	Immunology	Gilbert Jay, Ph.D.
Kathleen Newell	University of Kansas	Phillip Nelson, M.D., Ph.D.	Neuroscience	
Carmen Parrott	University of California (UCSF)	Al Rabson, M.D.	Immunology	Malcolm Martin, M.D.
*David Pezen	Loyola-Stritch School of Medicine	Roscoe Brady, M.D.	Neuroscience	Malcolm Martin, M.D.
*Michael Rees	University of Michigan	Henry Metzger, M.D.	Immunology	Alfred Singer, M.D.
Jesus Rivera-Nieves	University of Puerto Rico	Al Rabson, M.D.	Immunology	Brian Murphy, M.D
Mitchell Rosner	Harvard Medical School	Arthur Nienhuis, M.D.	Cell Biology and Regulation	Louis Staudt, M.D., Ph.D.
Scott Simpson	University of Texas, Galveston	Flossie Wong-Staal, Ph.D.	Molecular Genetics	Joe Bolen, Ph.D.
*Spencer Smith	Duke University	Arthur Nienhuis, M.D.	Molecular Genetics	Michael M. Gottesman, M.D.
David Tanen	New York University	Phillip Nelson, M.D., Ph.D.	Neuroscience	Steven Paul, M.D.
*Michelle Verplanck	Michigan State University	Phillip Nelson, M.D., Ph.D.	Neuroscience	Jin Kinoshita, Ph.D.
Joseph Vinetz	University of California (UCSD)	Thomas Kindt, Ph.D.	Neuroscience	Louis Miller, M.D.
Kathleen Waite	Duke University	Henry Metzger, M.D.	Immunology	Paul Plotz, M.D.
Jill Watanabe	Johns Hopkins University	Michael Brownstein, M.D., Ph.D.	Immunology	Barry J. Richmond, M.D.
David Weiner	SUNY at Buffalo	Michael Brownstein, M.D., Ph.D.	Neuroscience	Mark R. Brann, Ph.D.
Keith Wharton	University of Arizona	Flossie Wong-Staal, Ph.D.	Neuroscience	Ulrich Siebenlist, Ph.D.
Jane Wong	Tulane University	Arthur Nienhuis, M.D.	Molecular Genetics	Jeffrey Rubin, M.D., Ph.D.
Victor Wu	Johns Hopkins University	Henry Metzger, M.D.	Cell Biology and Regulation	Henry McFarland, M.D.
Stephanie Telesetsky Young	Duke University	Michael Brownstein, M.D., Ph.D.	Immunology	

*Second year

Continued

HHMI-NIH MEDICAL STUDENTS 1989–1990

Scholar	*School*	*Advisor*	*Program*	*Preceptor*
Nicholas Abidi	University of Pennsylvania	Joram Piatigorsky, Ph.D.	Molecular Genetics	Mark Bolander, M.D.
Lisa Airan	Northwestern University	Joram Piatigorsky, Ph.D.	Molecular Genetics	Michael Gottesman, M.D.
*Kirsi Allison	Dartmouth Medical School	Thomas Waldmann, M.D.	Immunology	Warren Strober, M.D.
Ann Arthur	Yale University	Bruce Howard, M.D.	Molecular Genetics	John Nickerson, Ph.D.
*Stephen Boorstein	University of Michigan	Thomas Waldmann, M.D.	Immunology	David H. Sachs, M.D.
James Butrynski	Wayne State University	Gilbert Ashwell, M.D	Cell Biology and Regulation	Allen Spiegel, M.D.
Lawrence Chin	University of Massachusetts	Alan Rabson, M.D.	Immunology	Ada M. Kruisbeek, Ph.D.
Christopher Chow	Johns Hopkins University	Joram Piatigorsky, Ph.D.	Molelcular Genetics	Robert Nussenblatt, M.D.
Jodi Cohen	Washington University	Gilbert Ashwell, M.D.	Cell Biology and Diagnosis	Lee Eiden, Ph.D.
Anita Dash	Northeastern Ohio	Rachel Myerowitz, Ph.D.	Molecular Genetics	Peggy Zelenka, Ph.D.
Emad Eskandar	University of Southern California	Roscoe Brady, M.D.	Neuroscience	Barry J. Richmond, M.D.
*Elizabeth Farr	University of Michigan	Thomas Waldmann, M.D.	Molecular Genetics	Bruce Weintraub, M.D.
Michael Ferrick	University of Michigan	Thomas Kindt, Ph.D.	Immunology	Chi-Chao Chan, M.D.
*David Frucht	Duke University	Henry Metzger, M.D.	Molecular Genetics	Malcolm Martin, M.D.
Sara-Jo Gahm	University of Vermont	Alan Rabson, M.D.	Immunology	Louis A. Matis, M.D.
John Gilstad	Uniformed Services University of Health Sciences	David Davies, Ph.D.	Structural Biology	Eduardo Padlan, Ph.D.
Heidi Hagman	Oregon Health Sciences University	Michael Brownstein, M.D., Ph.D.	Neuroscience	Allen Spiegel, M.D.
Hirofumi Hashimoto	Stanford University	Thomas Kindt, Ph.D	Immunology	Ronald H. Schwartz, M.D.
*Carol Ann Huff	Baylor College of Medicine	Rachel Myerowitz, Ph.D.	Molecular Genetics	Stuart Yuspa, M.D.
Suzanne Jan de Beur	George Washington University	Michael Brownstein, M.D., Ph.D.	Neuroscience	Ronald G. Crystal, M.D.
Michael Johnson	University of Virginia	Alan Rabson, M.D.	Immunology	Rachel Caspi, Ph.D.
Sang-Mo Kang	Harvard Medical School	Henry Metzger, M.D.	Immunology	Michael J. Lenardo, M.D.
*Robert Kanterman	University of Miami	Gilbert Ashwell, M.D.	Cell Biology and Regulation	Julius Axelrod, Ph.D.
Stamatina Kaptain	Harvard Medical School	Rachel Myerowitz, Ph.D.	Molecular Genetics	Richard D. Klausner, M.D.
James Kirby	University of Pennsylvania	Rachel Myerowitz, Ph.D.	Molecular Genetics	Susan Gottesman, Ph.D.
David Koeplin	University of Michigan	Bruce Howard, M.D.	Molecular Genetics	Michael Blaese, M.D.
Andrew Li	George Washington University	Joram Piatigorsky, Ph.D.	Molecular Genetics	Jeffrey M. Hoeg, M.D.
*Ajit Limaye	University of Washington	Phillip Nelson, M.D., Ph.D.	Neuroscience	Eric Ottesen, M.D.
Paul Lin	University of Mississippi	Bruce Howard, M.D.	Molecular Genetics	John Minna, M.D.
*William Lipham	Baylor College of Medicine	Arthur Nienhuis, M.D.	Immunology	Igal Gery, Ph.D.
David McDermott	University of Virginia	Michael Frank, M.D.	Immunology	Harry Malech, M.D.
Matthew Mitchell	Harvard Medical School	Roscoe Brady, M.D.	Molecular Genetics	Steven Paul, M.D.
*Kathleen Newell	University of Kansas	Phillip Nelson, M.D., Ph.D.	Neuroscience	Edward Ginns, M.D., Ph.D.
Susan Nicholson	University of Pittsburgh	Roscoe Brady, M.D.	Neuroscience	Howard Nash, M.D., Ph.D.
Shaila Patel	Ohio State University	David Davies, Ph.D.	Structural Biology	John Weinstein, M.D., Ph.D.
Andrew Phillips	Yale University	David Davies, Ph.D.	Structural Biology	Lawrence Samelson, M.D.
Roberto Pineda II	University of Minnesota	Thomas Kindt, Ph.D.	Immunology	Gerald J. Chader, Ph.D.
Leslie Reynolds	Duke University	Henry Metzger, M.D.	Cell Biology and Regulation	Ilan R. Kirsch, M.D.
*Mitchell Rosner	Harvard Medical School	Arthur Nienhuis, M.D.	Molecular Genetics	Louis Staudt, M.D., Ph.D
Tanya Rutledge	Harvard Medical School	Michael Frank, M.D.	Immunology	Richard D. Klausner, M.D.
Todd Seidner	University of California, Los Angeles	Henry Metzger, M.D.	Immunology	Arnold Rabson, M.D.
John Stahl	Duke University	Alan Rabson, M.D.	Immunology	Lance A. Liotta, M.D., Ph.D.
*David Weiner	State University of New York	Michael Brownstein, M.D., Ph.D.	Neuroscience	Mark R. Brann, Ph.D.
Rex Wong	University of Michigan	Michael Frank, M.D.	Immunology	Steve Rosenberg, M.D., Ph.D.
Samuel Wu	Harvard Medical School	Michael Brownstein, M.D., Ph.D.	Molecular Genetics	Jonathan Ashwell, M.D.

*Second year

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PUBLICATIONS OF HHMI-NIH RESEARCH SCHOLARS 1988–1989

Books and Chapters of Books

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The Institute's grants program began in 1987, with the first awards being announced in October 1987. The initial focus of the grants program has been on graduate and undergraduate education in the biological sciences, with future program development planned in public and precollege science education and health sciences policy.

GRADUATE EDUCATION IN THE BIOLOGICAL SCIENCES

In the first two years of the grants program, graduate education activities have been aimed at expanding the pool of biomedical investigators through the awarding of individual fellowships. In addition, a small number of grants have been awarded to selected institutions that traditionally have played a unique national role in education and research training in the biological sciences.

Doctoral Fellowships in the Biological Sciences

To respond to future needs for biomedical research scientists and educators, the Institute has established a doctoral fellowship program in the biological sciences. Fellowships are awarded for full-time study toward a Ph.D. or Sc.D. degree in the biological sciences that parallel the Institute's five research programs. Each fellowship provides an annual stipend for up to five years, as well as an annual cost-of-education allowance provided to the institution at which the fellow will study. The fellowships are intended for students at the beginning of their graduate study, including foreign nationals as well as United States citizens. Students who hold or are currently pursuing degrees in medicine, veterinary medicine, or dentistry also may apply for fellowships for study toward the Ph.D. degree.

Panels of distinguished biomedical scientists, under the auspices of the National Research Council (NRC) of the National Academy of Sciences (NAS), which administers the fellowship competition on behalf of the Institute, evaluated 1,100 eligible applications in the 1989 competition. Of these, 61 fellows were selected by the Institute. The new fellows, who are drawn from some 45 undergraduate institutions (including 8 from outside the U.S.), will do their graduate work at 26 academic institutions across the country. The number of doctoral fellows now totals 120, with 34 universities serving as fellowship institutions. The Institute anticipates awarding a similar number of new fellowships each year, until approximately 300 fellows are supported in any given year.

Medical Student Research Training Fellowships

To strengthen and expand the pool of medically trained researchers, a new fellowship program was launched in the fall of 1988. This program of Medical Student Research Training Fellowships enables selected medical students who have developed an interest in fundamental research during the course of their medical studies to spend an intensive research year in a laboratory. Building on the successful Research Scholars Program (the joint effort of the Institute with the National Institutes of Health in Bethesda), the new fellowship program will provide an opportunity for up to 60 fellows each year to engage in full-time research at any medical school, university, or research institute in the United States.

On the basis of review by a panel of eminent academic scientists and physicians, the Institute named 47 medical student fellows in 1989, the first year of the program. The fellows, drawn from 24 medical schools, will be pursuing their research training at 21 academic institutions in the United States.

The fellowship provides a stipend to the fellow, a research allowance for the student's mentor, and an allowance to the fellowship institution. Each year a small number of these fellows, as well as Research Scholars, will be selected for up to two years of continued fellowship support upon return to their medical studies.

Research Resources

Through its research resources program, the Institute provides support to research and educational organizations that serve as unique national resource laboratories and teaching facilities, including those that provide biological stocks and materials. The initiative focuses on support for organizations that serve the biomedical research community as a whole and whose activities relate to the established medical research programs of the Institute. Two new awards were made in 1988, to the Marine Biological Laboratory in Massachusetts and to the NAS/NRC Institute of Laboratory Animal Resources

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in Washington, D.C. Earlier multiyear awards to the Cold Spring Harbor Laboratory in New York and to The Jackson Laboratory in Maine continued into 1988.

A $4 million grant to the Marine Biological Laboratory supports its training and education programs for a period of seven years. In addition to supporting summer courses in neuroscience, physiology, embryology, and microbiology, the award provides for planning and initiating new projects in electronic information storage, retrieval, and management by the Marine Biological Laboratory/Woods Hole Oceanographic Institute Library. A summer pilot program demonstrated a variety of electronic databases. In addition, computer networks were established to link the library and teaching facilities.

A $500,000 three-year grant to the Institute of Laboratory Animal Resources supports a number of activities related to animal models and genetic stocks for biomedical research and education. Grant-supported activities in the first year included attention to policy and procedure concerns regarding use and preservation of transgenic strains of animals. The Institute of Laboratory Animal Resources provides information and advice to the federal government, the national and international scientific communities, and the general public. It publishes standard reference documents on the care and use of laboratory animals, maintains a directory of sources, and convenes expert groups to consider relevant scientific and policy issues.

The ongoing $7 million grant to the Cold Spring Harbor Laboratory supports the development of a new neuroscience facility and an expanded program of advanced courses in neuroscience and structural biology for a period of three years. Institute funds have enabled the Laboratory to embark on a series of intensive two-week courses given in the spring and fall to complement its usual summer program, which is partially supported by the grant as well.

The $2 million grant to The Jackson Laboratory, over a three-year period, supports the acquisition of scientific equipment for training and education, the enhancement of the genetic resource program, and renovation of an educational facility. Among the educational activities supported by the grant were an international conference on transgenic mice in MHC (major histocompatibility complex) research and a summer course on preservation of frozen embryos. Grant funds also have been used for expansion of facilities whereby laboratory mice bred outside this laboratory may be brought in without danger of infection to the resident mouse colonies. In addition, renovation of a residential facility for students has begun.

New Graduate Programs

As another avenue to strengthen the pool of medical trained researchers, plans were developed during the year for a fellowship program to be launched in the fall of 1989. This program of Postdoctoral Research Fellowships for Physicians will support physicians seeking full-time training in fundamental research. The 25 fellowships awarded annually will provide three years of support, including stipends and research and institutional allowances. Physicians who have completed at least two years of postgraduate clinical training and no more than three years of research training are eligible to compete, including United States citizens and foreign nationals.

UNDERGRADUATE EDUCATION IN THE BIOLOGICAL SCIENCES

Undergraduate science education has been characterized in numerous national reports as the essential bridge linking secondary schools with the nation's graduate and professional schools that train future investigators, physicians, and science faculty.

To help strengthen undergraduate science education and enable it to fulfill its traditional role, the Institute has established an Undergraduate Biological Sciences Education Initiative. This initiative has two goals: first, to encourage and provide opportunities for undergraduate students—especially minority group members and women—to prepare for graduate studies and careers in biomedical education, research, or medical practice and second, to enhance the overall quality of education and research in biology and related disciplines by helping undergraduate institutions bring fresh perspectives to teaching and by developing new programs in emerging areas.

Other principal objectives include broadening the academic base of biology by more closely integrating the teaching of biology with chemistry, physics, and mathematics; firmly grounding biology students in these disciplines; and strengthening the ties between undergraduate institutions and secondary schools to help improve biology and related science curricula at the secondary level.

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In 1988 the Institute awarded grants to 44 private liberal arts colleges and historically black institutions in an initial round of grants competition. Subsequently in 1988 the Institute invited 101 public and private universities (selected on the basis of their records in preparing students for medical school or for doctorate degrees in biology and related disciplines) to submit grant proposals. An external advisory panel of distinguished academic scientists evaluated the proposals, and in June 1989, grants were awarded to the following 51 institutions:

Brown University, R.I.
Carnegie Mellon University, Pa.
Case Western Reserve University, Ohio
City College of the City University of New York
College of William and Mary, Va.
Colorado State University
Columbia University, N.Y.
Cornell University, N.Y.
Dartmouth College, N.H.
Duke University, N.C.
Emory University, Ga.
Indiana University
The Johns Hopkins University, Md.
Lehigh University, Pa.
Louisiana State University
Massachusetts Institute of Technology
Miami University, Ohio
New York University
Ohio State University
Pennsylvania State University
Princeton University, N.J.
Purdue University, Ind.
Rensselaer Polytechnic Institute, N.Y.
Rice University, Tex.
Stanford University, Calif.
Stevens Institute of Technology, N.J.
Tufts University, Mass.
University of Arizona
University of California, Davis
University of California, Irvine
University of California, San Diego
University of California, Santa Cruz
The University of Chicago, Ill.
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, Rio Piedras
University of Southern California
University of Texas at Austin
University of Utah
University of Vermont
University of Virginia
University of Washington
University of Wisconsin–Madison
Wayne State University, Mich.
Yale University, Conn.

PUBLIC AND PRECOLLEGE SCIENCE EDUCATION

Through its grants program, the Institute plans also to explore avenues of support for public and precollege science education. A study of high school biology education by the Board on Biology of the Commission on Life Sciences of the NAS/NRC is supported by an Institute grant. The study, directed by a committee of scientists and educators, is focusing on curricula and teaching. Activities include a review of past efforts to improve high school biology programs and current efforts to improve precollege science education through such approaches as magnet schools and science high schools. A report of the study, with recommendations for future action, will be issued in the winter of 1990.

Grants program development planned for 1990 will be based, in part, on that report and on the reports of relevant projects under the Undergraduate Biological Sciences Education Initiative.

HEALTH SCIENCES POLICY PROGRAMS

The Institute also has plans for a program in the general area of health sciences policy and bioethics. These initiatives may focus on the public understanding of science, the impact of technology on society, innovation in research and its assessment, and the various roles of the public and private sectors in public policy related to science and technology.

In support of these goals, in 1987 the Institute awarded a grant to the Institute of Medicine (IOM) of the NAS. Over the next several years the grant supports the monitoring of advances in the health sciences and preparation of annual briefings to identify opportunities and needs in research areas of special significance. In addition, a special focus group (Technological Innovation in Medicine) is addressing policy questions concerning technology

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transfer. A series of workshops will be devoted to examining aspects of the transfer process from scientific inception to development, testing, and application. The third area of grant-supported activities is that of anticipating legal and ethical issues that may arise from the revolution in biology and advances in medicine. A study is under way to scrutinize how public understanding of the relevant science and technology affects decision-making about health issues.

Studies and reports from the IOM will be used to help guide the Institute's development of future grants programs in this area.

PROGRAM ASSESSMENT

The Institute is committed to a comprehensive program of assessment that will focus initially on the outcomes of grants in graduate and undergraduate science education. The Institute also will be monitoring trends in science education and resources for science, using established national databases. Assessment activities are central to the Institute's grants program and will augment established programs and help to guide future planning.

The commitment to the assessment program must necessarily be long-term. Shifts in national or institutional trends are frequently slow and incremental. Furthermore, certain outcomes of interest, such as a fellow achieving a faculty-level appointment, may require a 10- or 15-year interval subsequent to the initial fellowship award. Nonetheless, many short-term outcomes are of equal interest.

Many science education data collected by and for a number of federal agencies, including the Department of Education, the National Institutes of Health, and the National Science Foundation, are organized into an integrated system of databases called CASPAR (Computer-Assisted Science Policy Analysis Research). For example, nonconfidential national figures on enrollments, degrees awarded, and sources of support are available and will be used in the Institute's program of assessments. Comprehensive science education databases maintained by the NAS/NRC and the Association of American Medical Colleges (AAMC) also are of interest, and will be used within limits set by confidentiality protections.

Under a five-year, $480,000 grant, the AAMC will track the training and careers of medical students who participate in relevant Institute programs. (Two programs offer support for a year of full-time fundamental research by medical students: the Medical Student Research Training Fellowships and the Research Scholars at NIH, the latter being a joint program with NIH.) To help place the Institute's programs in context, the AAMC will also track a general cohort of medical students, and, with the cooperation of NIH, will monitor careers of M.D./Ph.D. students supported through the NIH Medical Scientist Training Program. To obtain reliable national figures on the numbers of students studying toward combined M.D. and Ph.D. degrees, the AAMC will undertake special surveys of the medical schools and will use databases on the awarding of M.D. and Ph.D. degrees.

As part of its assessment activities, the Institute is undertaking a study of support for science education by private independent and corporate foundations and voluntary health associations. Initially the target of the study will be graduate, undergraduate, and precollege science education support. The Institute awarded a one-year, $55,000 contract to the Center for Health Policy Studies of Georgetown University to collect and analyze data in these areas and to submit a report to the Institute. The principal source of data for the study is the Foundation Center library, a national repository of information on foundation giving. In recognition of its assistance in providing data resources, as well as materials on private giving, the Institute awarded the Center a grant in the amount of $30,000 in 1989.

In addition, as noted in the Graduate Education section, the Institute awarded a grant totaling $404,000 to the NAS/NRC in 1989. This program assessment grant supports the evaluation of applications to the doctoral fellowships competition.

COMBINED ALPHABETICAL LISTING OF PUBLICATIONS BY THE SCIENTIFIC STAFF OF THE INSTITUTE 1988–1989

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1982	Richard D. Palmiter, Ph.D.
1983	Richard K. Gershon, M.D.
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