

Division of

Cancer Etiology

1986 Annual Report
Volume I

October 1, 1985-
September 30, 1986

U.S. DEPARTMENT
OF HEALTH
AND HUMAN SERVICES

National
Institutes of
Health

Bethesda,
Maryland 20892

National
Cancer
Institute (U.S.)

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ANNUAL REPORT
DIVISION OF CANCER ETIOLOGY

NATIONAL CANCER INSTITUTE

October 1, 1985 through September 30, 1986

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ANNUAL REPORT
OF THE
DIVISION OF CANCER ETIOLOGY
NATIONAL CANCER INSTITUTE

Richard H. Adamson, Ph.D., Director

October 1, 1985 through September 30, 1986

OVERVIEW

The Division of Cancer Etiology is comprised of three major programs: the Biological Carcinogenesis Program, the Chemical and Physical Carcinogenesis Program and the Epidemiology and Biostatistics Program. The Biological Carcinogenesis Program consists of one extramural component (the Biological Carcinogenesis Branch) and five intramural laboratories (the Laboratory of Cellular and Molecular Biology, the Laboratory of Molecular Oncology, the Laboratory of Molecular Virology, the Laboratory of Tumor Virus Biology and the Laboratory of Viral Carcinogenesis). The Chemical and Physical Carcinogenesis Program consists of two extramural components (the Chemical and Physical Carcinogenesis Branch and the Low Level Radiation Effects Branch) and eight intramural laboratories (the Laboratory of Biology, the Laboratory of Cellular Carcinogenesis and Tumor Promotion, the Laboratory of Chemoprevention, the Laboratory of Comparative Carcinogenesis, the Laboratory of Experimental Carcinogenesis, the Laboratory of Experimental Pathology, the Laboratory of Human Carcinogenesis and the Laboratory of Molecular Carcinogenesis). The Epidemiology and Biostatistics Program consists of one extramural component (the Extramural Programs Branch) and four intramural Branches (the Biostatistics Branch, the Clinical Epidemiology Branch, the Environmental Epidemiology Branch and the Radiation Epidemiology Branch).

The current structure of the Division reflects a major reorganization begun in 1983 and completed last year. This Division-wide reorganization should enhance interaction between intramural scientists and the extramural community, facilitate the sharing of scarce resources and generally improve the management of research in cancer etiology. Efforts continue to further increase interaction between the biological carcinogenesis and chemical carcinogenesis laboratories and to enhance their interaction with the components of the Epidemiology and Biostatistics Program. The current organizational chart for DCE is shown in Figure 1.

The distribution of funds among the intramural and extramural components of the Chemical and Physical Carcinogenesis Program, the Biological Carcinogenesis Program and the Epidemiology and Biostatistics Program is shown in Table 1 and Figure 2.

The past year has seen a continued reduction in overall contract support. This has been realized by gradual phase-out of contract-supported, investigator-initiated research in areas where grants provide adequate coverage, by reducing

activities which provide materials and services, and by initiating and continuing various cost-recovery mechanisms. For example, in the Biological Carcinogenesis Branch six resource contracts are functioning in the cost-recovery, or "payback" mode. These include two for production of viral reagents, two for animal resources, one for specialized testing services and one for storage and distribution of stored frozen biological reagents. In the Chemical and Physical Carcinogenesis Branch, payback systems have been established for the Radiochemical Repository and for the Chemical Carcinogen Reference Standard Repository. All samples distributed under the chemical research resource program are now under this cost-recovery system. Reimbursement for full or partial costs of services has led to a more careful use of costly resource reagents and chemicals, with a subsequent reduced level of effort in several resource contracts or the termination of unnecessary activities. As support for research contracts has dropped, support for investigator-initiated research grants has continued to increase. In addition, the Cooperative Agreement is now being utilized as an additional instrument of support. The Biological Carcinogenesis Branch, in particular, has utilized this mechanism, and now administers 26 Cooperative Agreements at a level of 2.33 million dollars in support of research on the acquired immune deficiency syndrome (AIDS), HTLV infection, and bovine leukemia virus. In addition to its intramural and extramural research, the Division has been involved in other activities during the past year which merit attention. These are:

1. Frederick Cancer Research Facility (FCRF)

Research conducted by the contractor began in the early 1970s as a series of unrelated tasks or projects. Today, the carcinogenesis program at the FCRF has gradually evolved into a coordinated effort on the mechanisms of action of viral and chemical carcinogens and has been transferred to the Office of the Deputy Director, NCI (Dr. Peter Fischinger), which coordinates all FCRF activities. FCRF is also the focus of NCI research on AIDS, with particular emphasis on the development of an AIDS vaccine. As a separate effort, the contractor provides support services and materials to intramural investigators who work in NCI laboratories at the Frederick facility. Government and contractor scientists have combined their efforts to create a center of excellence for cancer research. At the present time the following DCE laboratories are located at FCRF: the Laboratory of Comparative Carcinogenesis, the Laboratory of Experimental Pathology, the Laboratory of Molecular Oncology, and the Laboratory of Viral Carcinogenesis.

2. The DCE Board of Scientific Counselors

Chartered in 1978, the Board is an advisory body whose members are drawn from the scientific community outside NIH. Chosen for their recognized expertise in chemical carcinogenesis, molecular biology, viral oncology, chemoprevention, epidemiology, immunology, pathology and genetics, the members of the Board advise the Division Director on a wide variety of matters which concern the progress of the Division's programs.

One particularly important responsibility of the Board has been the examination of the productivity and performance of staff scientists by site visit review of the intramural laboratories. These visits have been conducted by teams which, as a rule, are comprised of two to four members of the Board and four or more investigators from the scientific community outside NIH whose special fields of expertise match those of the scientists in the Laboratory or Branch being reviewed. The site visit reports, which reflect a consensus of the members of the team, are critically examined by the entire Board of Scientific Counselors and, after discussion, recommendations based on the reports are submitted to the Division Director. Approximately one year later the Division Director reports back to the Board on the changes made as a result of the site visit.

The second cycle of site visits to the Division's entire intramural operation began with a site visit to the Environmental Epidemiology Branch on May 18-20, 1983. The Laboratory of Molecular Virology was site visited on December 2, 1983; the Laboratory of Biology on March 1984; and the Clinical Epidemiology Branch on April 12-13, 1984. The site visit to the Laboratory of Cellular and Molecular Biology occurred on July 17-19, 1984 and the Laboratory of Molecular Carcinogenesis was site visited on September 13-14, 1984. The site visits to the Laboratory of Cellular Carcinogenesis and Tumor Promotion occurred on February 21-22, 1985; to the Laboratory of Chemoprevention on May 30-31, 1985; to the Laboratory of Molecular Oncology on June 13-14, 1985 and to the Laboratory of Comparative Carcinogenesis on July 11-12, 1985. The Laboratory of Human Carcinogenesis was site visited on November 21-22, 1985, and the site visit to the Laboratory of Experimental Pathology took place on March 10, 1986. The most recent site visit, to the Laboratory of Experimental Carcinogenesis, occurred on March 20-21, 1986. During the coming year site visits will be made to three laboratories (the Laboratory of Tumor Virus Biology, the Laboratory of Viral Carcinogenesis and the Laboratory of Molecular Virology) and three branches (Biostatistics Branch, Environmental Epidemiology Branch and Radiation Epidemiology Branch).

Another important function of the Board is that of concept review, in which the members pass judgment on concepts for grant- or contract-supported activities. The Board continued to examine new concepts this year as new directions in extramural research activities developed.

Several workshops, involving Board members as well as participants from the scientific community outside NIH, were held this year. As a consequence, new initiatives resulted in issuing and/or funding Requests for Applications (RFAs) in the areas

of transformation mechanisms of human polyoma viruses; transformation mechanisms of papillomaviruses; development and assessment of retroviral vaccines; novel exogenous and endogenous human retroviruses; National Collaborative Chemoprevention Projects; mutagens in human food; neoplasia in finfish and shellfish; involuntary exposure to tobacco smoke; dietary markers for epidemiologic studies of cancer; and development, validation and application of biochemical markers of human exposure for use in epidemiologic studies.

A workshop on papillomaviruses held during the past year was chaired by a member of the DCE Board of Scientific Counselors and the recommendation of the participants was that the DCE issue an RFA to stimulate further study of the mechanisms by which these human viruses transform human cells and their effects on cellular differentiation and the immune response. The Division also issued two Program Announcements during the past year. One, entitled "The Biological Role of Exocyclic Nucleic Acid Derivatives in Carcinogens," was developed as a result of a workshop on the subject co-sponsored with the International Agency for Research on Cancer. Another recently issued Program Announcement concerns basic mechanistic studies on the role of omega-3 polyunsaturated fatty acids in cancer prevention.

The objectives of the extramural research programs are accomplished through a variety of extramural mechanisms including traditional individual research project grants (R01), program project grants (P01), new investigator awards (R23), conference grants (R13) Cooperative Agreements (U01), contracts (N01), small business innovative research (SBIR) awards (contract [phases I and II], N43 and N44; grant [phases I and II], R43 and R44), academic enhancement awards (R15) and outstanding investigator awards (R35). A new NIH-wide mechanism called Method to Extend Research in Time (MERIT) Award was initiated during this fiscal year.

Continuing modifications of funding mechanisms approved by the Board include a gradual transfer of current resources to a cost-reimbursement system (payback system), increased availability of funding to support new resource activities, the phasing out of contract-supported research in areas adequately covered by grant applications, an increased use of RFAs to stimulate research activity in high priority areas, and the use of the Cooperative Agreement as an additional mechanism of support for investigator-initiated research involving significant involvement of NCI staff.

The Division is most grateful to the members of the Board for giving so generously of their time, effort, and expertise in helping to guide the future of the Division's programs. There is every expectation that the Board will continue to play a vital role as the Division's foremost outside advisory group.

FIGURE 1

DIVISION OF CANCER ETIOLOGY

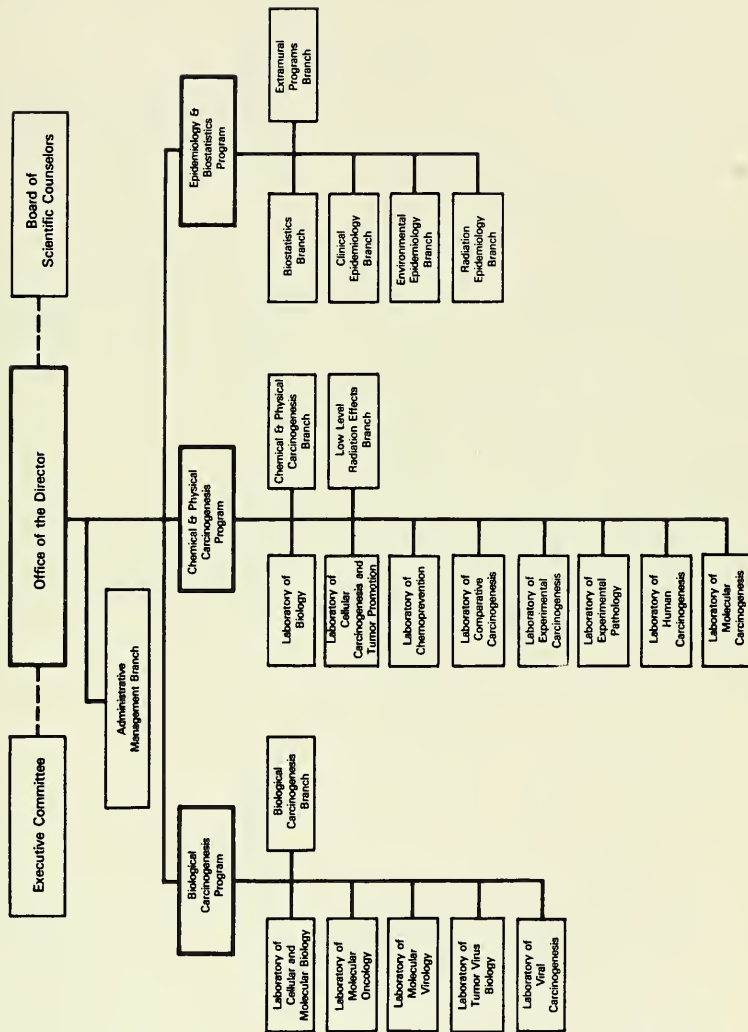


TABLE 1

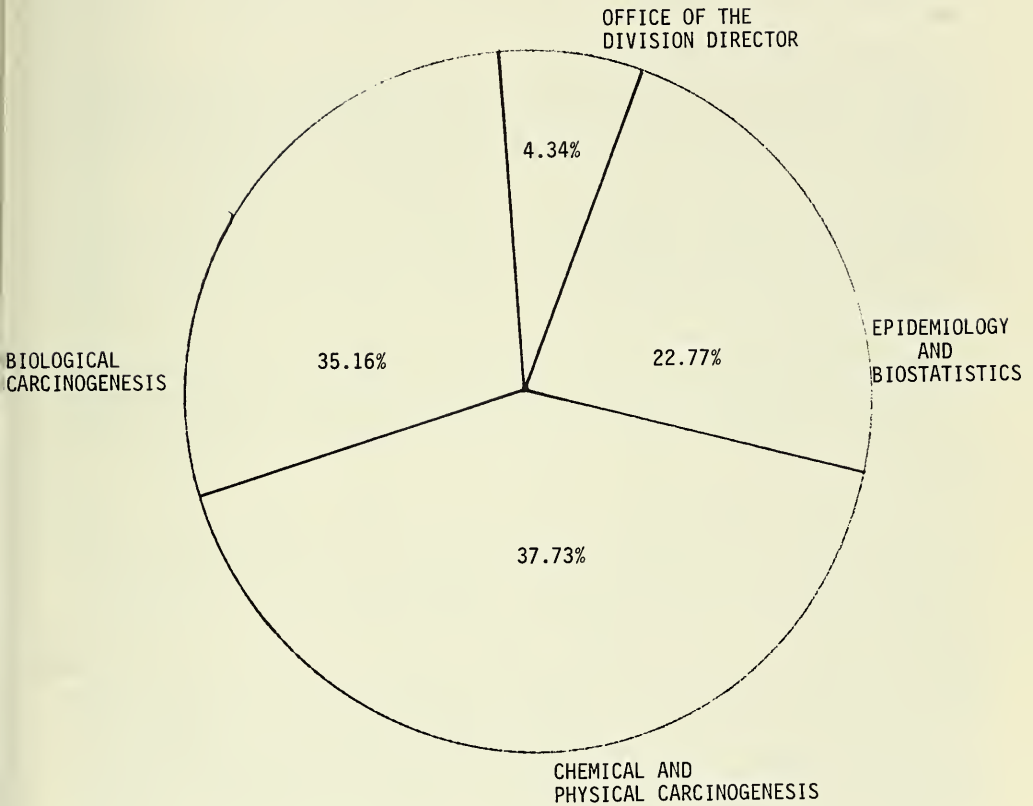
NATIONAL CANCER INSTITUTE
DIVISION OF CANCER ETIOLOGY

Table of Mechanisms by Organizational Unit Based on
Estimated Current Level of Expenditures
(Dollars in Thousands)

FY 1986 Estimate

	<u>Office of the Division Director</u>	<u>Chemical and Physical Carcinogenesis</u>	<u>Biological Carcinogenesis</u>	<u>Epidemiology and Biostatistics</u>	<u>Total</u>
Inhouse	4,821	15,183	12,182	8,081	40,267
Contracts	5,326	7,650	2,452	19,000	34,428
RFA	0	4,204	3,363	3,040	10,607
Cooperative Agreements	606	1,152	2,338	585	4,681
Research Project Grants	0	65,363	66,839	25,762	157,964
Total	<u>10,753</u>	<u>93,552</u>	<u>87,174</u>	<u>56,468</u>	<u>247,947</u>

FIGURE 2
NATIONAL CANCER INSTITUTE
DIVISION OF CANCER ETIOLOGY
Current Distribution of Funds
FY 1986 Estimate



SCIENTIFIC HIGHLIGHTS

Introduction: The Division of Cancer Etiology is responsible for planning and conducting the Institute's program of coordinated research on cancer causation and basic research on prevention. The Division supports both intramural laboratories and branches and extramural programs which seek to elucidate the mechanisms of cancer induction at each step of the cellular process from initiation to transformation of normal cells into malignant cells. The overall purpose of these studies is to provide information for reversing, interrupting or preventing this process before the development of clinical disease.

To accomplish these goals, investigators pursue fundamental studies on normal and malignant cells and on carcinogens or procarcinogens, such as viruses and chemicals, using the disciplines of cellular and molecular biology, immunology, biochemistry, and microbiology. Epidemiologic studies of human populations are performed to identify risk factors predisposing to various cancers using the disciplines of clinical medicine, genetics, mathematics, and biometry.

Excellent model systems are available to scientists studying the effects of exposure to a diversity of potentially carcinogenic factors in the environment. Much fundamental information has been obtained by studying tumor viruses which induce cells to become malignant. These viruses provide tools for understanding how cellular genes and their products convert a normal cell into a tumor cell. Investigations in the area of biological carcinogenesis have shown that viral information present as nucleic acid sequences (oncogenes) in normal and malignant cells and replicating with them may be intimately involved in the development of cancer. Triggered by chemical carcinogens, radiation, hormones, aging factors and other influences, these highly conserved viral sequences may direct the synthesis of proteins responsible for malignant transformation of the cell.

Similarly, chemical carcinogens, both naturally occurring and synthetic, provide a diverse group of chemicals with cellular and tissue selectivities that influence transformation and progression from the normal to the malignant state. Studies in the area of chemical/physical carcinogenesis cover a broad range of approaches with emphasis on the mechanisms of action of chemical and physical agents. Investigators have focused on the effects of carcinogens on cell structure and function, the relationships between molecular structure and carcinogenic activity, enzyme systems associated with the metabolic activation of procarcinogens to carcinogenic forms, the effects of the binding of carcinogens to DNA, systems that repair DNA damage and on the possible activation of oncogenes by chemical carcinogens. Other relevant efforts include investigation of the role of various factors in the environment of the cell; for example, promoters, hormones and growth factors which may be required for the progression of the "initiated" cell to the malignant state.

Finally, studies on the natural history of cancer in humans and on the incidence of cancers in different geographic locations help to identify causal associations of intrinsic and extrinsic risk factors with various cancers. In view of the increasing importance of nutrition and life-style in the causation or prevention of cancer, in particular the role of macro- and micronutrients in

the diet, alcohol consumption and smoking, special emphasis has been given to projects that may have more immediate health implications. Many studies deal with the presence of mutagens, carcinogens and natural anticarcinogens in foods; other studies assess the carcinogenic effects of smokeless tobacco and passive smoking. In addition, major studies on the viral etiology of cancer, cancer incidence in the workplace, effects of low-level radiation, and environmental pollutants in air, water, and soil are under investigation.

Biological Carcinogenesis

Oncogenes

A primary emphasis of many ongoing investigations concerns RNA tumor viruses. These viruses are unique among animal viruses in their mode of transmission and the intimate association that has evolved between these agents and cells of a large number and wide variety of vertebrate species. The etiologic role of this virus group has been established for naturally occurring cancers of many species, including some subhuman primates. Certain members of this virus group, so-called "replication-defective" transforming viruses, have risen by a recombination with cellular genes or proto-oncogenes. As such, these viruses offer an unparalleled opportunity to elucidate the processes by which such genes cause malignancies, and research to elucidate the processes involved in malignancy is actively being pursued utilizing RNA tumor viruses as models.

Recent investigations have provided strong evidence that proto-oncogenes are also frequent targets of genetic alterations leading human cells along the pathways to malignancy. Much current research is aimed at identifying genes that can be implicated as oncogenes in human cancer, their molecular mechanisms of activation, and the mode of action of their translational products.

As noted above, acute transforming retroviruses have risen in nature by substitution of viral genes necessary for replication with discrete segments of host genetic information. When incorporated within the retroviral genome, such transduced cellular sequences, the oncogenes, acquire the ability to induce neoplastic transformation. Some of the same cellular genes or proto-oncogenes have also been implicated as important targets for genetic alterations that may lead normal cells to become malignant independent of virus involvement.

Members of the ras gene family are activated as oncogenes in many different cancers. The availability of molecular clones of the normal and activated alleles of human ras proto-oncogenes made it possible to determine the molecular mechanisms responsible for the malignant potential of these genes. The genetic lesions responsible for activation of a number of ras oncogenes have been localized to single base changes in their p21 coding sequences. Considerable effort has been directed at elucidating the structure and function of the p21 products. For example, deletion of small sequences from the viral H-ras gene have been generated, and resulting ras p21 mutants have been expressed in E. coli. Purification of each deleted protein allowed the in vitro characterization of GTP binding, GTPase, and autokinase activities of the proteins. Microinjection of the highly purified proteins into quiescent NIH 3T3 cells, as well as transfection experiments utilizing an LTR-containing vector, were utilized to analyze the biological activity of the deleted proteins. Two

small regions, located at 6-23 and 152-165 residues, were shown to be absolutely required for in vitro and in vivo activities of the ras product. By contrast, the variable region comprising amino acids 165-184 were shown not to be necessary for either in vitro or in vivo activities. Thus, it was demonstrated that (1) amino acid sequences at positions 5-23 and 152-165 of the p21-ras protein are probably directly involved in the GTP-binding activity, (2) GTP binding is required for the transforming activity of ras proteins and, by extension, for the normal function of the proto-oncogenic product, and (3) the variable region at the C-terminus end of the ras p21 molecule from amino acids 165 to 184 is not required for transformation.

Next evaluated was whether decreased in vitro GTPase activity is uniformly associated with ras p21 mutants possessing efficient transforming properties. Normal H-ras p21-[Gly¹²-Ala⁵⁹] as well as an H-ras p21-[Gly¹²Thr⁵⁹] mutant exhibited in vitro GTPase activities at least fivefold higher than either H-ras p21-[Lys¹²-Ala⁵⁹] or H-ras p21-[Arg¹²-Thr⁵⁹] mutants. Microinjection of as much as 6×10^6 molecules/cell of bacterially expressed normal H-ras p21 induced no detectable alterations of NIH 3T3 cells. In contrast, inoculation of $4-5 \times 10^5$ molecules/cell of each p21 mutant induced morphologic alterations and stimulated DNA synthesis. Moreover, the transforming activity of each mutant expressed in a eukaryotic vector was similar and at least 100-fold greater than that of the normal H-ras gene. These findings establish that activation of efficient transforming properties by ras p21 proteins can occur by mechanisms not involving reduced in vitro GTPase activity.

Using a gene transfer assay, it has been possible to demonstrate consistent activation of a c-ras gene in clinical samples of human chronic myelogenous leukemia (CML) from both chronic and blast crisis phases of the disease. If confirmed by further work, this finding would represent the first time that abnormalities of a ras gene have been found consistently in one type of human malignancy.

The v-sis transforming gene encodes the woolly monkey homolog of human PDGF-2. After its synthesis on membrane-bound polyribosomes, the glycosylated precursor dimerizes in the endoplasmic reticulum and travels through the Golgi apparatus. At the cell periphery, the precursor is processed to yield a dimer structurally analogous to biologically active PDGF. Small amounts of two incompletely processed forms are detectable in tissue culture fluids of simian sarcoma virus (SSV) transformants. However, the vast majority remains cell-associated. Thus, this growth factor-related transforming gene product is not a classical secretory protein. These findings define possible cellular locations where the transforming activity of the sis/PDGF-2 protein may be exerted. A scheme for partial purification of biologically active v-sis-coded protein from cells transformed with SSV has made possible a functional comparison of the transforming protein with PDGF. The SSV-transforming gene product is capable of specifically binding PDGF receptors, stimulating tyrosine phosphorylation of PDGF receptors, and inducing DNA synthesis in quiescent fibroblasts. Each of these activities was specifically inhibited by antibodies to different regions of the v-sis gene product. Moreover, viral infection of a variety of cell types revealed a strict correlation between those cells possessing PDGF receptors and those susceptible to transformation by SSV. These findings provide

evidence that SSV-transforming activity is mediated by the interaction of a virus-coded mitogen with PDGF receptors.

The structure of the normal human *c-sis*/PDGF-2 transcript was determined by a combination of cDNA cloning, nuclease S1 mapping and primer extension. Nucleotide sequence analysis revealed that the 3373-nucleotide, *c-sis*/PDGF-2 mRNA, contained only a 723-bp coding sequence for the PDGF-2 precursor polypeptide. The coding sequence was flanked by long 5' (1022 bp) and 3' (1625 bp) untranslated regions. The 5' noncoding region, as well as upstream flanking genomic sequences, contained clusters of specific short repeat sequences. The 3' noncoding sequence lacked the highly conserved polyadenylation signal. A consensus transcriptional promoter sequence, TATAAA, was identified 24 base pairs upstream of the mRNA start site and an enhancer-like TG element was detected about 180 bp downstream from the site of polyadenylation. These findings identify putative regulatory elements of the *c-sis*/PDGF-2 gene.

cDNA molecules representing the complete coding sequence of a new human gene have been isolated. This gene, designated *c-slk*, is a member of the *src* family of oncogenes. Nucleotide sequence analysis revealed that this gene encoded a 537-residue protein which was 86% identical to the chicken proto-oncogene product, p60^{c-src}, over a stretch of 191 amino acids at its carboxy terminus. In contrast, only 6% amino acid homology was observed within the amino terminal 82 amino acid residues of these two proteins. It was possible to activate *c-slk* as a transforming gene by substituting approximately two-thirds of the *c-slk* coding sequence for an analogous region of the *v-fgr* onc gene present in the Gardner-Rasheed feline sarcoma virus. The resulting hybrid protein molecule expressed in transformed cells demonstrated protein kinase activity with specificity for tyrosine residues.

DNA of a human diffuse B-cell lymphoma induced an unusual transformed focus on NIH 3T3 cells. The transforming gene was serially transmissible, conferred the neoplastic phenotype to NIH 3T3 cells, and appeared to be larger than 20 kbp by analysis of transfectants for conserved human DNA sequences. From a cosmid recombinant DNA library of a third-cycle transfectant, overlapping clones spanning 80 kbp of cellular DNA were isolated. One clone, which contained a 45-kbp insert comprised entirely of human sequences, was shown to be biologically active, with a specific transforming activity of 650 focus forming units/pmol. By restriction mapping and hybridization analysis, this human transforming gene, designated *dbl*, was unrelated to any previously reported oncogene.

The cellular gene encoding the receptor for epidermal growth factor (EGF) has considerable homology to the oncogene of avian erythroblastosis virus. In a human mammary carcinoma, a DNA sequence was identified that is related to *v-erbB* but amplified in a manner that appeared to distinguish it from the gene for the EGF receptor. Molecular cloning of this DNA segment and nucleotide sequence analysis revealed the presence of two putative exons in a DNA segment whose predicted amino acid sequence was closely related to, but different from, the corresponding sequence of the *c-erbB*/EGF receptor. Moreover, this DNA segment identified a 5-kb transcript distinct from the transcripts of the EGF receptor gene. Thus, a new member of the tyrosine kinase proto-oncogene family has been identified on the basis of its amplification in a human mammary carcinoma.

Major advances in recent studies on raf oncogenes include the isolation, sequence determination, chromosomal mapping and characterization of biological activity of novel raf genes, A-raf-1 from mouse and man and A-raf-2 from man. This increases the number of human raf-related genes to four, two of which, c-raf-1 and A-raf-1, are active genes. All of these genes are relevant to human pathology, c-raf-1 and A-raf-1 because their product has transforming activity; and c-raf-1 is implicated in a variety of human carcinomas on cytogenetic and biochemical grounds. C-raf-2, located near the top of the short arm of chromosome 4, has several restriction enzyme polymorphisms associated with its flanking DNA and, thus, has become a useful clinical marker for a hereditary disease, Huntington's chorea. A-raf-2, while more distant than another active oncogene (met) to the site of the cystic fibrosis locus on chromosome 7, may still be useful in further characterizing this region. Experiments to functionally map c-raf-1 and A-raf-1 products in the signal transduction pathway of growth factors have shown that both genes appear to act downstream of ras oncogenes, placing them at the end of a chain which includes most other peripheral cytoplasmic and membrane-associated oncogenes. Thus, raf genes and their regulation would appear to be ideal targets for the tailoring of modulating drugs with a potential for therapeutic significance. The c-raf-protein-associated ser/thr-specific kinase was further characterized. N-terminal truncation appears to increase kinase activity, as well as transforming ability. There is a division of labor between members of the raf family: c-raf-1 appears to have a basic regulatory role in most tissues, i.e., it is expressed everywhere, albeit at varying levels, whereas A-raf-1 is highly restricted in its expression with highest levels in the epididymis. Biochemical evidence was obtained for a role of c-raf-1 in all histological types of lung carcinoma. The gene is expressed at unusually high levels in 60-80% of all tumors as determined by Northern, immunoblot and immunohistochemical techniques. The role of c-raf in transformation of these cells is currently being evaluated. To aid in this evaluation, an animal model system was developed for rapid (5-13 weeks), high frequency induction of lung adenocarcinoma in mice. The tumors contain transforming DNA as judged by DNA transfection and express uniformly high levels of c-raf RNA and protein. In fact, the levels are well in excess of those seen in NIH 3T3 cells transformed by an LTR-driven c-raf-1 cDNA construct. Live cell fluorescence indicated surface fluorescence of the predominantly cytoplasmic c-raf protein. Attempts to modulate tumor induction and promotion in these mice by stimulation of an anti-raf protein-directed immune response yielded promising results since the latency, but not the final evidence of tumor development in mice promoted with BHT, was almost doubled.

It has been demonstrated that the avian leukemia virus, E26, has homologous sequences in mammalian species which are dispersed to two different chromosomal loci that have distinctive domains; these have been termed ets-1 and ets-2 and correspond to the 5' and 3' regions of the v-ets oncogene, respectively. In humans these loci have been mapped to chromosome 11 for ets-1 and to chromosome 21 for ets-2 by somatic cell hybrid studies and direct in situ analysis using isotopically-labeled probes. By in situ hybridization of an ets-2 clone to normal human chromosome preparations, the assignment of human ets-2 to chromosome 21 was confirmed and regionally localized to the HSA 21q22.1-22.3 portion. Similarly, using a characterized panel of mouse and hamster hybrids it has been possible to assign the ets-1 and ets-2 to the murine chromosomes 9 and 16, and with the feline hybrids assigned the proto-oncogenes ets-1 and

ets-2 to the feline chromosomes D1 and C2, respectively. Unlike Mammalia, the chicken proto-oncogene for ets was contiguous, indicating that both ets-1 and ets-2 are situated on the same chromosome and are not dispersed. From overlapping chicken clones, v-ets oncogene-related exons were found to be dispersed over 35 kb of the avian genomic DNA. From sequenced portions of the human ets-1 and ets-2 clones an extremely strong (over 90%) homology of predicted amino acid residues compared to the avian and viral oncogenes was demonstrated. The murine ets-2 is essentially identical to the proto-ets-2 gene of humans. The ets-2 homology between humans and Drosophila, as well as humans and sea urchin, is also in excess of 90% at the predicted amino acid level. The high levels of amino acid homology observed for the ets genes, the highest thus far noted for any proto-oncogenes, suggests that these genes--from widely separated, evolutionarily-diverse species--must perform important functions to be so stringently conserved. The two genetically distinct loci are transcriptionally and differentially active in human cells yielding distinct products; they also appear to be independently regulated. We have prepared synthetic oligopeptides corresponding to a conserved predicted amino acid sequence in the ets-2 gene and prepared monoclonal antibodies against this synthetic peptide. Such antipeptide antibodies are able to specifically immunoprecipitate p135gag-myb-ets protein from E26 infected cells. In addition, at least one of these antibodies precipitates a p56 ets-2 protein from cells.

The use of a polyclonal antibody prepared against a highly-conserved region of the ets-2 (a region found in all three ets-2 transcripts) identified the p56 protein from a human cell line and in the mouse thymus. Similarly, polyclonal antibodies prepared against the v-ets-2-expressed protein also immunoprecipitates a p56 protein; the one-dimensional peptide maps of the p56 proteins detected with the antipeptide antibody and the antibody against the v-ets-expressed protein are identical. Using standard subcellular fractionation techniques, immunoblotting and immunoprecipitation techniques, it was found that the ets-2 p56 is localized in the nucleus. These investigations have made it possible to localize the ets genes relative to a number of chromosome breakpoints characteristic of specific translocations occurring in neoplastic cells. The ets-1 gene is located on a very narrow region of chromosome 11, between the breakpoints of the t(4;11)(q21;q23) of an anti-leukemia and the t(11;22)(q24;q12) of a Ewing's sarcoma. The ets-2 gene on chromosome 21 is activated by the breakpoints of a t(8;21)(q22;q22) of an acute myelogenous leukemia (AML-M2) and a t(21;22)(q22;q11) of a chronic myeloid leukemia. Both ets-1 and ets-2 were not found to be rearranged using probes representing the 3' regions of the genes.

To characterize the MH2 virus containing the dual oncogenes, mht and myc, and to elucidate the nature and functional contributions of each, deletion and frameshift mutants of each of the two MH2 genes, v-mht and v-myc, were constructed. Studies on these mutants indicated that the v-myc gene transformed avian primary cells in vitro by itself, without requiring the second potential oncogene. The v-mht gene did not show detectable transforming ability in vitro but may enhance transformation of primary avian cells in cooperation with the v-myc gene. A replication-defective murine retrovirus carrying the v-myb and v-ets oncogenes was also constructed. DNA sequence analysis verified that the

insertion was in frame with the first 34 codons of murine gag p15. This constructed virus (ME26) encodes a murine gag-avian gag-myb-ets fusion protein of approximately 133 kilodaltons. Following cotransfection into NIH 3T3 cells with pSV2neo, G418-resistant colonies were selected. Thirteen of 18 isolated colonies were found to contain integrated ME26 sequences. Significantly, recombinant ME26 virus was rescued from the supernatant of transfected cells upon superinfection with a murine helper virus. The rescued ME26 virus could be successfully passed into NIH 3T3 cells by infection, without gross alterations of the recombinant genome. In fact, a protein of about 133 kilodaltons was detected in NIH 3T3 cells transfected with the ME26 construct. This protein reacted specifically with the anti-avian gag and anti-ets antisera and may represent an authentic murine-avian viral fusion protein.

The nucleotide sequence of the human locus of the newly described N-myc proto-oncogene has been obtained. The gene is closely related to c-myc and is expressed in a controlled manner during the course of mouse embryogenesis and in a restricted manner in adults, particularly in neural tissues. Amplification and over-expression of N-myc are common abnormalities in the advanced stages of human neuroblastoma; these measurements of N-myc appear likely to be useful in the diagnosis and management of this disease. The product of N-myc has been identified; it is a nuclear protein that has a short half-life and binds to DNA, properties consistent with membership in the myc gene family. Additionally, it has been shown that N-myc can transform cultured cells in two settings: rat embryo cells in cooperation with mutant c-Ha-ras and established rodent fibroblasts without assistance. Both forms of transformation require vigorous expression of N-myc, but no intrinsic abnormality of the gene. These findings establish N-myc as an authentic proto-oncogene and strengthen the suspicion that its amplification can contribute to the progression of human neuroblastoma. Molecular constructs containing "anti-sense" RNA have been made which allow stable repression of gene expression in cultured cells. This work was first accomplished in pilot studies in which herpesvirus thymidine kinase was the repressed gene and has now been extended to work with N-myc in the hope of obtaining direct evidence to implicate this gene in tumorigenesis.

To study myc genes under inducible control, human myc genes containing entire coding sequences or only the second and third exons were cloned and expressed under the control of the metallothionein promoter, using a bovine papillomavirus (BPV) vector system. Permanent cell lines expressing human myc proteins have been established. Analysis of human myc gene products in these cell lines indicates that: (i) myc gene products enhance BPV-induced transformation, (ii) 62 to 64-kd human myc protein is made either when all three exons are present or only when second and third exons are present, (iii) human myc protein expressed in mouse cells is mainly compartmentalized in the nucleus, (iv) human myc protein is inducible with heavy metal ions, and (v) though the myc gene is present on an episome in the cell, it appears to be subject to similar regulatory control mechanism(s) like those controlling the endogenous c-myc gene.

The application of new techniques in cell genetics, molecular biology, linkage analysis and in situ hybridization has resulted in the identification and characterization of over 1500 human loci, a value which now exceeds the number of genes mapped in Drosophila. Efforts have been focused on somatic cell hybrid panels and in situ hybridizations to genes related to neoplastic

processes including (1) cellular proto-oncogenes, (2) growth factors, (3) growth factors and receptors, (4) endogenous retroviral families, (5) integration sites for retroviruses, and (6) restriction genes that delimit retrovirus replication in mammals. Within the last few years, the human gene map has experienced a large increase in the number of neoplasia loci that have been mapped to specific chromosomal positions. Of the 35 specific human loci that have been chromosomally mapped to date, 13 (40%) have been assigned by DCE intramural scientists and their collaborators. This year, efforts have been concentrated on understanding the genomic organization of several genes: rel, ets, trk, tpr, fms, met and endogenous retroviral families. Three of these genes, ets, met-tpr, and trk, were found to be composite genes derived from the fusion of chromosomally disparate functional loci. Truncation of these cellular genes in a variety of human neoplasias, as well as in certain nonneoplastic pathologies (e.g., ets-2 in Down's syndrome or met in cystic fibrosis) which were suggested by their chromosomal position, are under investigation. The emerging gene map of neoplasia-associated loci continues to provide an unprecedented opportunity for molecular genetic analysis of the initiation and progression of neoplastic processes.

Control of Gene Expression

Efforts to elucidate the signals associated with gene expression have continued, with particular emphasis on regulatory events which take place at the level of transcription and processing of RNA. Elucidation and analysis of novel genetic elements, enhancer sequences, which appear to be responsible for controlling the rate at which particular genes are transcribed, are in progress. The existence of these enhancer sequences was demonstrated not only in the genomes of DNA viruses such as SV40, JCV and BKV, but also in the long terminal repeats (LTRs) of retroviruses. Using a combination of in vivo and in vitro assays, it has been shown that enhancer sequences often exhibit host-cell specificity, and thus may be among the elements involved in controlling the host range of certain viruses as well as the tissue-specific expression of certain eukaryotic genes. A number of laboratories have now shown that enhancers are critical elements in determining the activity of eukaryotic genes and that they function in a tissue- or organ-specific fashion. A major effort will be directed at determining whether or not enhancer sequences play a role in the developmental and tissue-specific regulation of gene expression. In addition, regions of enhancer elements will be mutagenized to elucidate those sets of nucleotides associated with the general activation phenomenon as well as the cellular specificity. In vivo and in vitro experiments have been designed in an attempt to examine the mechanism by which the activator/enhancer sequences function. Current experiments are focused on defining and characterizing the biological macromolecules which interact with these regulatory elements.

A particularly fruitful avenue of investigation has involved the development of transgenic mice. Using JC virus as a model system, it has been possible to establish lines of mice with specific neurologic defects including a dysmyelination syndrome and tumors of the adrenal medulla. Animals have also been established which carry "foreign" class I histocompatibility antigens (see below). These will be important in studying a number of basic principles

associated with immunosurveillance and the distinction between foreign and self.

An understanding of the structure and function of the class I histocompatibility antigens (the classical transplantation antigens) and in particular, the roles of these cell-surface antigens in relation to the neoplastic state has been a subject of considerable interest. These studies are of singular importance because the ability of the immune system to identify and destroy tumor cells depends upon their presentation by the class I antigens to the cytotoxic T-lymphocytes. A major goal is to obtain an understanding of the factors which govern immune recognition of foreign cells. Attempts are directed at in vivo and in vitro immune modulation which will hopefully enhance the ability of the host to recognize tumor cells as "foreign" and to eliminate them by immunologic means.

Molecular cloning and identification of class I loci has led to the finding of a gene that encodes a soluble or secreted class I-related antigen. Because of variations in the level of expression of this gene in different inbred mouse strains and an unusual tissue-restriction in its expression, it was suggested that this soluble histocompatibility antigen, represented as a serum protein--perhaps a tolerogenic form of the class I antigens, could act as a blocking factor to regulate the function of cytotoxic T-cells in the process of immune surveillance. Studies are in progress to test this hypothesis by using the secreted class I antigen to modulate T-lymphocyte recognition of tumor cells.

Studies Related to HTLV and AIDS

Recent research has demonstrated that HTLV-III/LAV is part of a large family of lymphotropic retroviruses found throughout the world in man, primates, and other mammals. Characterization of the various members of this family of viruses and their mechanism(s) of pathogenesis should facilitate the development of preventive and therapeutic measures for this disease.

Rhesus monkeys at the New England Regional Primate Research Center suffer from a fatal immunodeficiency disease similar to human AIDS. Investigators at this Center, in collaboration with others at Harvard University, have isolated a retrovirus which is serologically related to the human AIDS virus, HTLV-III/LAV. The simian virus, which is designated simian T-cell lymphotropic virus III (STLV-III), can be experimentally transmitted to other monkeys in which it causes a fatal immunodeficiency disease. The existence of this new AIDS-like animal model may facilitate the development and testing of preventive and therapeutic approaches against the human AIDS virus in a readily available primate.

Recent studies have shown that a virus antigenically related to simian T-cell lymphotropic virus (STLV-III) causes natural infections in wild-caught African green monkeys in Africa, in the apparent absence of disease. It was found that healthy humans in Senegal, West Africa are infected with a virus which is more closely related to the African green monkey virus (STLV-III_{AGM}) than to other HTLV/LAV/HIV viruses. They designated this human virus as HTLV-IV.

This unique model of silent and persistent infection by a "nonpathogenic" member of the AIDS family of retroviruses may provide information on the biological interaction of this family of retroviruses with primate hosts, including humans, and is thus important in the study of the origin of human AIDS and the development of a vaccine against AIDS.

Immunoglobulin produced from large pools of plasma has been used successfully to protect susceptible patients from HTLV infections by providing passive immunity. Sixty-three sera obtained from 23 patients with primary immunodeficiency syndromes between 1980 and 1986 were tested before and immediately after immunoglobulin infusion. Using Western immunoblots, 52 sera were HTLV-III/LAV antibody positive. There was a clear correlation between the amount of antibody present after infusion and the titer of antibody in the immunoglobulin lot administered. Since these patients cannot produce antibody, the data are consistent with an exogenous origin of the antibody and suggest that a positive titer for HTLV-III/LAV antibody in such patients is not the result of active infection with AIDS virus.

A DNA segment containing 82% of the *sor* open reading frame of HTLV-III/LAV virus was inserted into a prokaryotic expression vector, pJL6. The bacterially-synthesized *sor* protein reacted with sera from individuals infected with HTLV-III/LAV, indicating that *sor* was expressed as a protein product that was immunogenic *in vivo*. Antibodies to the purified, bacterially-synthesized *sor* protein immunoprecipitated a 23-kilodalton protein in HTLV-III/LAV-infected H9 cells, suggesting that this protein may be the *sor* gene product. In order to determine the cellular location of the *sor* protein, HTLV-III/LAV-infected H9 cells were fractionated into cytoplasmic, membrane, and nuclear fractions. The 23-kd protein detected by patient antisera was found to be present only in the cytoplasmic fraction. Several eukaryotic vector constructs were engineered to express the envelope genes of the HTLV-III/LAV virus. All such constructs, after cotransfection into TK⁻ cells along with the thymidine kinase gene, gave rise to selective transformants that enabled permanent lines to be established. Eight out of 10 such cell lines were found to be expressing HTLV-III/LAV envelope-specific mRNA. Four of these cell lines were expressing very high levels of message specific for the HTLV-III/LAV envelope gene. Recombinant vectors containing metallothionein promoter inducible HTLV-III envelope genes have recently been constructed containing SV40 T-antigen splice and polyadenylation signals; they are being introduced into embryonic mice for transgenic expression studies.

Bovine leukemia virus (BLV), with the human T-cell leukemia viruses (HTLV-I and HTLV-II), constitute a unique subgroup of RNA tumor viruses; they share a similar genetic organization, promoter architecture, and common strategy for regulating gene expression. As a group, these viruses are generally quiescent in their host; however, infected lymphocytes often become virus producers when transferred to *in vitro* culture conditions. Moreover, cell lines have been infected *in vitro* and often become virus positive. It was previously shown that the BLV promoter unit, contained in the proviral LTR, was transcriptionally inert in all cell lines except those which were productively infected with BLV. The *cis*-acting sequences in the LTR (adjacent to the promoter) which were required for transcriptional activity were defined by deletion mapping and by construction of chimeric promoter units. The CAT system was used to monitor gene expression and promoter activity. The BLV LTR contained sequence elements both upstream and downstream of the RNA start site that were required

for optimal gene expression. The upstream element behaved like a condition-specific enhancer element (or response element, see below) which was active only in cells productively infected with BLV. The downstream sequence element was found to increase gene expression when located 3' from the RNA start site of several promoter units and in all cell types, irrespective of BLV infection. BLV, like HTLV-I and HTLV-II, possesses several open reading frames in the pX region, located between the envelope gene and the 3' LTR. To determine whether the expression of the BLV pX genes results in transcriptional transactivation of their LTRs, uninfected cells were cotransfected with a plasmid containing the CAT gene controlled by the BLV LTR and expression plasmids containing BLV pX coding sequences. Synthesis of CAT enzyme, directed by the BLV LTR, is detected only when an active pX is present in target cells reflecting the transactivation induced or produced by pX genes. Although the mature, spliced pX mRNA has the potential to code for at least two proteins of ca. 38,000 and 17,000 MW, it was found that expression of the larger protein is necessary and sufficient to produce the transactivation effect. To define the sequence elements in the LTR that respond to the immediate effects of pX expression, uninfected cells were cotransfected with a BLV pX expression plasmid and plasmids containing the CAT gene controlled by portions of the BLV LTR or chimeric promoters containing both BLV and SV40 promoter elements. By *in vitro* gene swapping experiments, the target of pX action in the LTR was found to be composed of at least two pX response elements. The first is located within a region between 100bp and 170bp upstream of the RNA start site, and another is located immediately upstream of the TATA box. It is interesting to note that both HTLV-I and HTLV-II LTRs possess several short repeated sequences that, while not homologous to the BLV sequences, may play an analogous role in transcriptional regulation.

Lentivirus studies. Lentiviruses are a subfamily of retroviruses etiologically associated with arthritis, progressive pneumonia and slow neurological diseases in certain species. Relatively little is known about their genome structure, mechanisms of pathogenesis or evolutionary relationships with other subfamilies of retroviruses. In an effort to better understand the mechanisms by which these viruses induce such a variety of chronic diseases, the genomes of CAEV and equine infectious anemia virus (EIAV) were molecularly cloned and physically characterized. The latter, which bears some morphological similarity to the lentiviruses, has yet to be classified definitively as one. The nucleotide sequence of a highly conserved region within the CAEV and EIAV pol genes was determined. A much closer relationship of their predicted pol gene products to that of the presumed etiological agent of human AIDS than to those of other retroviruses was noted. Additional pairwise comparisons made it possible to generate an evolutionary tree showing that the pol genes of lentiviruses and oncoviruses have evolved from a common progenitor.

Studies on DNA Viruses

The transforming genes of small DNA tumor viruses, such as SV40 and mouse polyoma, appear to be very different from the oncogenes of the RNA retroviruses. These DNA oncogenes are totally of viral origin and produce proteins called tumor or "T" antigens which are necessary to the normal replication and maturation of these viruses in lytic infections. In the SV40 system, the large tumor antigen gene, which alone can transform cells, has been extensively

studied using biochemical, immunological, genetic and, most recently, transgenic methods. The polyoma middle T antigen has been identified as playing a central role in polyoma virus-induced oncogenesis. This protein is associated with a protein kinase activity which can be detected by *in vitro* phosphorylation of a tyrosine residue. This protein does not possess intrinsic protein kinase activity and is thought to associate with the cellular protein pp60c-src (the cellular homolog of the Rous sarcoma virus transforming gene). It is proposed that the polyoma virus middle T antigen protein kinase activity represents a property of the associated pp60c-src. The potential importance of this protein kinase activity in polyoma-mediated oncogenesis is suggested by the finding that viral mutants which are deficient in transforming potential also lack this associated kinase activity. Elevated levels of pp60c-src kinase activity have also been demonstrated in human tumor lines, particularly of neuroectodermal origin.

The role of pp60c-src in transformation has been extended from polyoma virus-transformed cells to human tumors. There is a 20- to 40-fold increase in pp60c-src tyrosine protein kinase activity in human neuroblastoma cell lines over that observed in either human glioblastoma cell lines or human fibroblasts. The levels of c-src RNA or pp60c-src protein in these cells, however, are not significantly elevated over those levels found in glioblastoma cells. Additional human tumor tissues and human tumor cell lines have been analyzed for the level of pp60c-src protein kinase activity. All cell lines derived from tumors of neuroectodermal origin which have a neural phenotype express high levels of the c-src tyrosine-specific protein kinase activity. In contrast, cell lines derived from tumors of neuroectodermal origin that do not express neural characteristics, such as glioblastomas and melanomas, have pp60c-src molecules with low levels of protein kinase activity. These results in the cell lines are parallel to the results seen in examining tumor tissues directly. Analysis of human tumor cell lines derived from tissues other than those of neuroectodermal origin are generally low with some notable exceptions. These exceptions include rhabdomyosarcoma, osteogenic sarcoma, Ewing's sarcoma, breast carcinoma, and colon carcinoma. Comparison of pp60c-src kinase activity in normal skeletal muscle and rhabdomyosarcoma tissue and in normal breast tissue and breast adenocarcinoma tissue revealed that pp60c-src activity was specifically elevated in the tumor tissues of both of these cases. These observations suggest that the phosphotransferase in some rhabdomyosarcomas and breast carcinomas may be a characteristic acquired during the malignant transformation of the cells.

Studies on the role of the papillomaviruses in human carcinogenesis and on the molecular biology of this group of viruses are being expanded. Studies on the bovine papillomavirus (BPV) type 1 have shown that the virus is able to transform rodent cells in tissue culture and as such provides a model for the systematic study of the molecular biology and genetics of this group of viruses. A transcriptional regulatory element within the 1 kb long control region (LCR) of the BPV-1 genome was mapped using an enhancer-dependent expression vector for chloramphenicol acetyltransferase. This enhancer element works in a position- and orientation-independent manner and its function is transactivated by the viral E2 gene product. The requirement for the E2 gene in viral transformation and replication assays was examined, and it was found that it is required for efficient cellular transformation and for stable plasmid maintenance. This function can be provided *in trans*. When the transformation functions are expressed from a surrogate promoter, however, the E2 gene product is no longer

required, indicating that its role in viral transformation is indirect. Its effect on transformation, as well as on stable plasmid replication, appears to be due to the transcriptional activation of an enhancer in the LCR which is required for the transcription of genes involved directly in transformation and replication. Mutagenesis studies have localized one of the two independent transforming genes of BPV-1 to the 3' half of the E5 ORF. The E5 transforming protein of BPV-1 has been identified utilizing an antiserum generated against a synthetic peptide corresponding to the 20-carboxy terminal amino acids of the E5 ORF. The E5 polypeptide is the smallest viral or cellular transforming protein yet defined. It is 44 amino acids in size and has an apparent molecular weight of 7 kd on SDS polyacrylamide gels. The transforming polypeptide is predicted to be strikingly hydrophobic with 68% of the amino acids being hydrophobic. Cell fractionation studies have localized this polypeptide primarily to cellular membranes. The polypeptide exists as a dimer within cells and has a half-life of approximately 2 hours.

Human papillomaviruses (HPVs) have recently been demonstrated to be strongly associated with cervical cancer and other anogenital malignancies. Investigators have identified HPV DNA in many pre-malignant, malignant and metastatic lesions in man. HPVs, therefore, are becoming an important area of tumor virus research. However, two major barriers to research on HPVs have been the lack of good model systems to study transformation and the lack of a permissive cell system to grow HPVs in the laboratory. A novel strategy has produced a mouse/human model which will allow virus replication, readily produce benign tumors, and may provide the basis of a true malignant transformation assay. The model uses normal human epidermal tissues (cervical, laryngeal, skin, or neonatal foreskin) which are exposed in vitro to a human condyloma (genital wart) extract containing HPV type 11. Small segments of these tissues are then placed under the renal capsule of nude mice. In the mice, the tissue exposed to condyloma extract develops into masses with all the histological and biochemical characteristics of a human condyloma, a benign tumor. All epidermal tissues tested in the mouse/human model showed evidence of this benign transformation, although there was a clear tropism for anogenital tissues. Interestingly, human neonatal foreskin produced the most extensive growths which practically effaced the mouse kidney. HPV-11 has been extracted from these transformed foreskin grafts. Sufficient virus was obtained to infect a new generation of normal neonatal foreskins. Thus, it will now be possible to produce HPV-11 and possibly other HPVs in the laboratory, ensuring an adequate supply for experimental purposes.

These results also demonstrate, for the the first time, that HPV can transform human tissue to a condylomatous state under laboratory conditions. Studies are now underway to extend this model to attempt to produce malignant transformation by using both serial passages of infected tissues in nude mice and treatment of the tissues with various chemical and viral cocarcinogens.

HPV-16 is another HPV implicated in the etiology of human cervical carcinomas. Investigators are beginning to elucidate the viral functions involved in transformation by the HPV family of viruses. Studies on HPV-16 DNA-containing cells have identified RNA transcripts for the E6 and E7 open reading frames both in established transformed human cell lines and in tissues from cervical carcinomas. The E7 transcripts were the most abundant transcripts in these transformed cells. These results suggest that the E7 and possibly the E6 gene products of

HPV-16 may be transforming proteins. Their persistence in established transformed cells over many generations tends to support a role for E7 and/or E6 proteins in the maintenance of the transformed state. It is expected that more HPV proteins will be identified and characterized in the near future.

Studies on transcriptional transactivation and control elements in HPV-16 have been initiated. A conditional transcriptional enhancer has been identified in the LCR of the HPV-16 genome. This element functions in an orientation- and position-independent manner to activate the enhancer deleted SV40 early promoter in the presence of the HPV-16 E2 gene product. Analyses have been done in acutely cotransfected monkey CV-1 cells in which the E2 gene of HPV-16 is expressed from the SV40 early promoter. Translational termination linkers cloned into the E2 ORF of the HPV-16 genome dramatically reduced the level of transcriptional transactivation. It has been shown that the analogous LCR enhancer element derived from BPV-1 can also be transactivated by the E2 gene product of HPV-16. Similarly, the HPV-16 LCR enhancer can be transactivated by the E2 gene product from BPV-1. It had previously been shown that two cervical carcinoma cell lines contain HPV-16 integrated into the host chromosome. The SiHa cell line contains a single copy of integrated HPV-16, and the CaSki cell line contains 600 copies of the integrated viral genome. The single HPV-16 genome in the SiHa cell line was cloned in a 10 kb HindIII fragment which also contained flanking cellular DNA sequences. The site of integration was determined with respect to the virus and it was found that the integration disrupted the E2 and E4 ORFs. A series of host viral fusion ORFs were noted but transcriptional studies indicated that these ORFs were not transcriptionally active. The HPV-16 genome had integrated into an Alu-repeated sequence as determined by the sequence flanking host sequences. Analysis of the three most abundant BamHI clones from the CaSki cell line revealed that these consist of: full length HPV-16 DNA, a 1.4 kb deletion of the LCR, and a 2.6 kb tandem repeat of the 3' transforming region or analogous region from the HPV-16 genome. Each of these abundant forms is repeated in a multiple head to tail tandem fashion within the genome. Northern blot analysis of the RNA from these two cervical carcinoma lines indicates that the HPV-16 genomes are transcriptionally active. In each case, utilizing subgenomic strand-specific probes, it has been demonstrated that the transcripts are derived primarily from the E6 and E7 ORFs.

Nucleic acid hybridization technology is being explored as a means of detecting the presence of pathogens not readily detected by classical virological techniques. EBV is an example of an agent that has proven difficult to recover from clinical samples, such as saliva. A new assay for the presence of EBV DNA was developed by using a cloned EBV DNA probe (BamHI-W fragment). The assay has good specificity and reasonable sensitivity. In contrast to the lymphocyte transformation assay, which is qualitative and takes 3 to 8 weeks to complete, the hybridization assay was semiquantitative and yielded results in 72 hours. The availability of this assay should facilitate assessment of the effects of radiation, chemotherapy, and antiviral agents on EBV in vivo and perhaps shed light on the possible role of the virus in the occurrence of monoclonal and polyclonal lymphoproliferative disease in immunocompromised patients.

Chemical and Physical Carcinogenesis:

In Vitro Studies on Human Tissue and Cells

Considerable progress has been made in the last few years in establishing conditions for culturing human epithelial tissues and cells. Normal tissues from most of the major human cancer sites can be successfully maintained in culture for periods of weeks to months. Chemically-defined media have been developed for long-term culture of human bronchus, colon, esophagus, and pancreatic duct. Primary cell cultures of human epithelial outgrowths have been obtained from many different types of human tissues. Isolated epithelial cells from human bronchus and esophagus can be transferred three or more times and can undergo more than 30 cell divisions. Human bronchial and esophageal epithelial cells can also be grown in serum-free culture medium. Morphological, biochemical and immunological cell markers have been used to identify these cells as unequivocally of epithelial origin. Clonal growth of normal human pleural mesothelial cells in a serum-free, as well as serum-supplemented culture medium, has also been achieved so that the in vitro transformation of these cells by asbestos can be studied.

The availability of nontumorous epithelial tissues and cells that can be maintained in a controlled experimental setting offers an opportunity for the study of many important problems in biomedical research, including carcinogenesis. For example, the response of human bronchial epithelial cells (enhanced growth or differentiation) after either (a) exposure to carcinogens and/or promoters or (b) DNA transfection by oncogenes is being actively investigated. Parallel investigations using epithelial tissues and cells from experimental animals allow investigators to study interspecies differences in response to carcinogens, cocarcinogens, and anticarcinogens.

Epithelial cells are of particular interest because most adult human cancers are carcinomas. As noted above, significant progress has been made in the past decade in developing methods for culturing human epithelial tissues and cells. Chemically-defined media have been developed for culturing normal human tissues and cells from organs with a high rate of cancer in humans. Serum-free media have several advantages in studies of cultured human cells, including (a) less experimental variability compared to serum-containing media; (b) selective growth conditions of either normal cells of different types (e.g., epithelial versus fibroblastic cells) or normal versus malignant cells; (c) identification of growth factors, inhibitors of growth, and inducers of differentiation; and (d) ease of isolating and analyzing secreted cellular products. Advances in cell biology, including the delineation of biochemical and morphological markers of specific cell types, have also facilitated the identification of cells in vitro (e.g., keratins as markers for epithelial cells and collagen types I and III for identifying fibroblasts).

Normal human bronchial epithelial cells (NHBE) were characterized with regard to DNA damage and repair resulting from environmental lung carcinogens. In most aspects, bronchial epithelial cells resembled bronchial fibroblasts. The O⁶-alkylguanine-DNA alkyltransferase repair system, as well as gamma, UV, and benzo(a)pyrene diol epoxide (BPDE) repair, were strikingly inhibited by the aldehyde class of lung carcinogens such as formaldehyde. Correspondingly, formaldehyde potentiated the mutagenicity of N-methyl-N'-nitrosourea. Formaldehyde also depleted cellular stores of protective thiols.

Cultured NHBE cells, human bronchial fibroblasts, and human pleural mesothelial cells are being used to study carcinogenesis induced by asbestos and related fibers at the cellular level. Electron microscopy studies demonstrate that asbestos fibers first attach to the cell membrane and are then phagocytosed into the cell. Even though all three cell types exhibited equivalent numbers of internalized fibers 24 hours post-exposure to the same concentration of amosite, the pattern of cytotoxicity of fibers in culture was found to parallel the selectivity of cell types as targets for carcinogenesis in vitro, e.g., asbestos and glass fibers were found to be 100 times more cytotoxic for the mesothelial cell and 10 times more cytotoxic for the NHBE cells than for the bronchial fibroblasts.

Because there is epidemiological evidence that tumor promotion plays a role in the development of human bronchogenic carcinoma, the effects of putative tumor promoters on NHBE cells are being investigated. The mouse skin tumor promoter 12-O-tetradecanoylphorbol-13-acetate (TPA) induces markers of terminal squamous differentiation in NHBE cells, including a squamous morphology change, increased plasminogen activator activity, cross-linked involucrin envelope (CLE) formation, and cessation of proliferation. In contrast, lung carcinoma cell lines are relatively resistant to the effects of TPA. These findings are consistent with the hypothesis that one mechanism of tumor promotion involves resistance of preneoplastic and neoplastic cells to inducers of terminal differentiation. Compounds representing two other classes of mouse skin tumor promoters, the polyacetates and the indole alkaloids, also induce terminal differentiation. Because cigarette smoke condensate (CSC) contains mouse skin tumor promoters, the effects of CSC and fractions thereof on NHBE cells were studied. None of the fractions was mitogenic over a broad range of concentrations. All of the fractions inhibited proliferation, with the most potent being the neutral methanol fraction (N_{meOH}). N_{meOH} also induced plasminogen activator activity, has the most pronounced effects on CLE formation, and was the most potent inhibitor of specific binding of epidermal growth factor. Based on these findings, it was concluded that the N_{meOH} fraction is the most likely to contain compounds with effects similar to those of the mouse skin tumor promoter.

It has been shown that transforming growth factor beta ($TGF-\beta$) is a mitogen for normal human mesothelial cells which do not produce this protein. In contrast, a mesothelioma cell line has been shown to produce $TGF-\beta$. Neither normal nor asbestos-modified mesothelial cells form tumors in athymic nude mice, while two mesothelioma lines tested do show this oncogenic potential. A model of autocrine stimulation and/or maintenance of mesothelioma cells by $TGF-\beta$ is suggested.

The major goal of another project is to detect and clone a transforming gene from the human prostatic cancer cell line, PC-3. Transforming activity has been found in PC-3 genomic DNA by the NIH 3T3 cell focus assay. Using the nude mouse tumorigenicity assay in conjunction with drug selection of NIH 3T3 cotransfected cells, the presence of an activated oncogene was confirmed. This unidentified oncogene, which does not appear to be a member of the ras family, is being cloned by sib selection using human repeated sequences as a probe. Preliminary results suggest the possible presence of suppressor DNA sequences that inhibit tumorigenicity of certain NIH 3T3 cells. Isolation of this suppressor activity also has been undertaken by sib selection. Autocrine activities of PC-3 and its highly metastatic variants are being investigated. An ascites line (PC-3asc) was isolated directly in serum-free medium from a

tumor obtained by inoculation of PC-3/mA2 in the nude mouse. The PC-3asc line was found to produce an autogenous factor that stimulated its own growth at low cellular inocula. In addition, preliminary data indicate that medium conditioned by PC-3asc cells has TGF activity in normal rat kidney (NRK) cells in soft agarose. This putative TGF is being isolated and will be further purified by standard biochemical procedures. Karyotypic analysis has shown that the PC-3asc line has many features in common with the parental line, PC-3/mA2, except for a few marker chromosomes with homogeneously staining regions, and numerous minute and double minute chromosomes.

A multi-pronged cocarcinogenesis approach is being utilized to overcome the genome stability and genetic suppression present in human cells. Papillomaviruses, known etiologic agents that cause benign proliferation of skin and mucosa, result in tumors that are histologically classified as papillomas or fibropapillomas. Because HPV-16 presence is also associated with human carcinomas, particularly genital cancer, and their tumor-derived cell lines, HPV-16 DNA has been transfected into human foreskin fibroblasts or epithelial cells to develop a model for studying cocarcinogenesis. The transfected cells are also useful for elucidating the molecular biology of HPV-16. A recombinant HPV-16 DNA (containing a head-to-tail dimer of the full length HPV-16 genome and a selectable marker, G418, that induces tumorigenic transformation of NIH 3T3 cells) was used to transfect foreskin-derived fibroblasts and keratinocytes by the calcium phosphate precipitation method. After G418 selection a cell population was obtained having increased saturation density and extended life span that is currently greater than 100 population doublings (PD) as compared to the control which senesced at about 50 doublings. Foci of piled-up cells formed in confluent cultures. Anchorage-independent growth in agarose was observable from PD 5 post-selection. The frequency of anchorage-independent colonies progressively increased with time in culture. Furthermore, X-irradiation (400R) of the transfected cells at PD 7 markedly accelerated morphologic transformation. Foci formation appeared 2 PD post X-irradiation; foci-derived cells were anchorage-independent (2%). Nontransfected x-rayed cells fail to form foci and senesced as did controls. Southern blot analysis of transfected cells (d0) and cells derived from an x-ray-induced focus (dX) showed the presence of HPV-16 sequences in multiple copies. Furthermore, both transformed d0 and dX express several HPV-16 mRNA species. Similarly, keratinocytes with an indefinite life span that possess HPV-16 and RNA expression have been developed. The keratinocytes are maintaining their nondifferentiated character *in vitro*. Thus, HPV-16 transfected human fibroblasts and keratinocytes provide a suitable model for studying molecular biology of HPV-16 and for cocarcinogenesis.

Higher levels of mutagens in the feces of certain populations eating a western diet have been shown to correlate with an increased risk of colon cancer. Ninety percent of this mutagenicity can be accounted for by a group of compounds called fecapentaenes, which are potent direct-acting mutagens in the Ames *Salmonella* assay. Although fecapentaenes may play a role in the etiology of human colon carcinoma, their genotoxic effects have not been previously studied in human cells. Fecapentaene-12 (fec-12), a prototype for this group of compounds, has been demonstrated to be cytotoxic, mutagenic, and to cause DNA single-strand breaks in cultured human fibroblasts. These results indicate that fec-12 is genotoxic in human cells and are consistent with the hypothesis that fecapentaenes may be involved in the pathogenesis of human colon cancer. Plasmid assays investigating the nature of fec-12 interactions with DNA have

shown that this compound causes interstrand DNA cross-links. Results from electron microscopic studies support these findings and also indicate that fec-12 directly causes DNA single strand breaks.

In Vivo Studies

A new category of metal binding proteins has been identified in rodent testicular tissue that are not metallothionein and whose capacity to bind the carcinogenic metal cadmium clearly is insufficient to prevent carcinogenesis in that organ by the carcinogenic metal. Recognition that this protein, previously reported to be metallothionein, is different removes a major discrepancy in the evolving picture of the role of metallothioneins in protection of specific tissues from carcinogenesis by the carcinogenic metals.

The induction of cancers from the epithelia of the different segments of the respiratory tract in animal models, by multifactorial mechanisms, is being studied by a treatment with combinations of chemical, physical and biological factors. Age at beginning of treatment was found to influence the induction of respiratory tumors by diethylnitrosamine in a segment-specific manner. In hamsters treated from birth, there is an increased incidence and decreased latency of nasal cancers; no significant age-related effect was found on the induction of tracheal or lung neoplasms. Concurrent intraperitoneal injection of dimethylsulfoxide increased the incidence and severity and decreased the latency of respiratory tumors induced by intratracheal administration of suspensions of benzo[a]pyrene (BP) combined with ferric oxide (Fe_2O_3). A complex multifactorial experiment on respiratory carcinogenesis in hamsters has been completed and the results are under analysis; preliminary results show that a single dose of N-methyl-N-nitrosourea (MNU) 2 weeks prior to a series of BP/ Fe_2O_3 administrations markedly enhances laryngeal and bronchial carcinogenesis relative to exposure to either carcinogen alone. The single instillation of MNU alone also induced a distant carcinogenic response (pancreas ductular carcinomas and adrenal cortical carcinomas). Localized injury to the trachea, characterized by increased epithelial mitotic activity, enhances the carcinogenic effect of BP/ Fe_2O_3 not only in the trachea, but also in the bronchi. Previous exposure to BP/ Fe_2O_3 increases DNA binding of BP in all exposed segments of the respiratory tract, with the trachea showing the highest and the most transient binding levels.

The increased incidence of malignant mesothelioma of the lung is associated with asbestos exposure. Therefore, chromosome analysis of mesotheliomas is relevant to characterizing this cancer. In an analysis of nine cases, all of which were examined prior to therapy, eight have a common chromosome alteration. The abnormality consisted of either interstitial or terminal deletions involving the long arm of chromosome 3. Interstitial deletions were observed in two cases: a deletion from the normally metacentric chromosome 3 to form a submetacentric and an inserted segment the size of a large band at 3p14-21 that restored the short arm, long arm ratio. In the six other cases, the deletion was terminal. The common abnormality at 3p14-21 may be critical to development of neoplasia. Specific alterations of the short arm of chromosome 3 (3p13-24) have also been detected in familial renal carcinoma, small cell carcinoma of the lung, mixed parotid gland tumors, B-prolymphocytic leukemia, ovarian carcinoma,

malignant lymphomas, and rhabdomyosarcoma. The involvement of this specific region in such diverse malignancies may be due to the presence, at 3p14-21, of a fragile site which is the most common in the human genome. At present no proto-oncogene is known to be located at 3p14-21. The possibility is raised that the 3p region may contain one or more "suppressor" or "regulatory" genes which could be either lost or inactivated.

Few investigations have studied possible adverse effects resulting from prolonged administration of chemopreventive agents. A recent discovery of potentially serious toxicity was made during the course of a study on the chemopreventive efficacy of the retinoid, 13-cis-N-ethylretinamide. The investigation was designed to determine the anticarcinogenic activity of this retinoid in the post-initiation period of butylhydroxybutylnitrosamine-initiated bladder cancer in the mouse. Three levels of carcinogen were administered to separate groups of mice which were subsequently given or not given dietary retinoid. Groups not receiving carcinogen, but administered dietary retinoid were also maintained. A most unexpected finding in these investigations has been the observation of a very high incidence of hepatocellular carcinomas, adenomas and other liver lesions by prolonged dietary administration of this retinoid. The liver cancer results from feeding the retinoid only; it does not appear to be tumor promotion following the nitrosamine initiation. In addition, "spontaneous" liver lesion frequency in noncarcinogen, nonretinoid, placebo-fed animals appears to be very low. Prolonged administration of 13-cis-NER (68 to 82 weeks) also appears to be necessary for induction of these liver lesions, since previous studies in this mouse model with this particular retinoid fed for only 22 weeks did not result in grossly-observable lesions.

Epidemiological studies have established that the oral users of snuff (snuff-dippers) face a significantly higher risk for cancer of the oral cavity, especially cancer of the gums, than nontobacco users. It has been established that snuff contains the carcinogen polonium-210 (0.2.2 pCi/g) and at least 12 carcinogenic N-nitrosamines. Of these, the tobacco-specific N-nitrosamines (TSNA) are the most carcinogenic and abundant ones, exceeding in concentrations by at least 3 orders of magnitude the upper limits set by the FDA for nitrosamines in consumer products. A study of snuff-dipping college students has demonstrated that the TSNA are extracted by saliva and that TSNA concentrations in saliva increase with length of habituation. Among the tobacco-specific nitrosamines, N¹-nitrososornicotine (NNN) and 4-(N-methyl-N-nitrosamino)1-(3-pyridyl)-1-butanone (NNK) are derived from nicotine by nitrosation and are powerful carcinogens. They are not only organ-specific carcinogens which induce cancer of the nasal cavity, esophagus and lung in mice, rats, and hamsters, but administration of NNN and NNK by oral swabbing also leads to oral tumors in rats. The TSNA are metabolically activated by alpha-hydroxylation to active species. Upon metabolic activation NNK is capable of forming O⁶-methylguanine, a promutagenic DNA adduct in vitro in both animal and human tissues and in vivo in animals. In a recent bioassay it was demonstrated that snuff, when repeatedly instilled in a surgically created canal in the lower lip of rats induces benign and malignant oral tumors, although the yield of tumors was not significant. Work is continuing with this model for determining effects of smokeless tobacco components.

Studies on Tumor Promotion

Studies in this area are carried out at the molecular level as well as in vitro and in animal models. The initiation event in skin carcinogenesis is highly correlated to an alteration in the program of terminal differentiation of epidermal cells. In cell culture, epidermal differentiation is regulated by the concentration of extracellular Ca^{2+} . Ca^{2+} concentrations which induce epidermal differentiation activate phospholipase C which catalyzes the metabolism of phosphatidylinositol. The consequent generation of diacylglycerol activates protein kinase C which serves as a second messenger in epidermal differentiation.

Most of the evidence for the role of protein kinase C in phorbol ester action is indirect. Its involvement in the mitogenic response of Swiss 3T3 cells was demonstrated directly by microinjection, using Swiss 3T3 cells down-regulated by chronic phorbol ester treatment. Emerging evidence suggests prominent proteolytic processing of protein kinase C. The catalytically active fragment of the enzyme was prepared by tryptic digestion and was characterized. Phospholipids regulated its activity in a pH- and substrate-dependent fashion, distinct from the activation of the intact enzyme by the interaction of phospholipids at the regulatory domain. The regulatory domain, being devoid of enzymatic activity and thus a pseudo-receptor, was also generated and is now being characterized. Analysis of protein kinase C modulators other than the phorbol esters afford unique insights into its function. Bryostatins, macrocyclic lactones, activate protein kinase C and compete for phorbol ester binding. Paradoxically, they blocked phorbol ester action in two differentiating systems (HL-60 promyelocytic leukemia cells and Friend erythroleukemia cells) and blocked some but not other responses in keratinocytes. In HL-60 cells, the bryostatins induced a similar but distinct pattern of phosphorylation. Diglycerides are the postulated endogenous analogs of the phorbol esters. In support of this hypothesis, treatment of mouse keratinocytes with bromooctanoic acid, which blocks triglyceride formation and causes diglyceride accumulation, induced effects similar to the phorbol esters. Bromooctanoic acid may provide a tool for assessing the tumor promoting activity of the diglycerides. Modeling studies comparing different classes of protein kinase C activators indicated putative common features. Novel structures incorporating these features inhibited phorbol ester binding and induced typical phorbol ester responses such as inhibition of EGF binding or phosphorylation of a 40 kd platelet protein.

Recent studies have shown that ultraviolet light, as well as chemicals, may be a cancer-promoting agent. Investigators have demonstrated that mouse skin cells can be initiated by x-irradiation, with tumors subsequently induced in these animals by ultraviolet light promotion. Particularly significant is the fact that after 16 fractions of x-rays (250 R, 2 x wk), ultraviolet light exposure (280-400 nm sunlamp) resulted in an incidence of more than 60% of squamous cell carcinomas under conditions where neither agent alone produced any tumors. Considering that x-rays plus the potent chemical 12-O-tetradecanoyl-13-acetate (TPA) resulted in an incidence of about 80% under the same conditions, it is likely that sunlight is also a potent promoter. These results are important when considering both therapeutic doses of x-irradiation and the risks associated with such doses followed by exposure to sunlight.

Tumor promotion in nonsquamous epithelia in rats and mice has been shown to be effected in a markedly specific pattern by long-acting sedative barbiturates and to involve the widest variety of unrelated epithelial tissue types known to be affected by tumor promoting agents. Promotion was demonstrable in thyroid follicular epithelium, liver parenchyma, renal cortical tubular epithelium, and urothelium, but was consistently demonstrated only in thyroid and liver, with effects in other tissues markedly dependent on the chemical structure of the barbiturate promoter. Short-acting hypnotic barbiturates, the kinds most commonly used in anesthesia in human beings, have not been found to promote; this good news, however, must be tempered by the demonstration that the benzodiazepine tranquilizers, diazepam and oxazepam, are as potent promoters for the mouse (but not the rat) liver parenchyma as is the barbiturate phenobarbital. Susceptibility to promotion has been shown to be a genetically determined trait that is inherited in a Mendelian dominant manner, and genetic analysis is in progress to quantify, locate, and ultimately to identify, clone, and sequence the genes that confer susceptibility to tumor promotion in specific target organs. A close correlation has been established between the capacity of agents to promote carcinogenesis in the liver and to induce biosynthesis of specific drug metabolizing enzymes of the P-450-dependent monooxygenase group, providing additional basis for the concept that tumor promoters act not simply by releasing a latent neoplastic cell from the constraints on growth conferred through intercellular communication, but through inductive processes on gene expression that serve also to alter the promoted cells from which tumors develop.

Studies on Oncogenes in Chemically-Induced Tumors

Significant advances have been made in studying the role of activation of specific oncogenes in the course of chemical carcinogenesis in certain rodent target tissues. The selective activation of K-ras oncogenes in the renal mesenchymal tumor of rats exposed once neonatally to methyl(methoxymethyl) nitrosamine has been confirmed and contrasts markedly with demonstration of the selective activation of an oncogene, neu (erbB2), in a high proportion of primary schwannomas of the peripheral, cranial, and spinal nerves induced by transplacental exposure to the direct-acting agent, N-nitrosoethylurea. This is in sharp contrast to the apparent absence of this gene in primary chemically-induced tumors of the central nervous system and to the sporadic and irregular detection of genes of the ras family in primary chemically-induced tumors of the rat liver and the rat intestine. In the systems which yield activated oncogenes in tumor tissues, the activated genes are never found in DNA preparations from grossly normal tissues of the same animal including liver, brain, or kidney. Also, in all cases, the presence of rat repetitive sequences in the transformed NIH 3T3 target cells has been consistent with the postulated role of ras genes in the transformation of the indicator cells. Consistent demonstration of specific dominant transforming genes in different tumor types, and their absence from others, argues against the random and artifactual activation of these transforming genetic sequences in the process of DNA isolation or in the transfection procedure and provides additional evidence in favor of the interpretation that they play a significant role in the process of carcinogenesis in those tissues in which they are consistently found. The successful application of the avidin-biotin peroxidase complex immunocytochemical technique to the localization and visualization of oncogene protein products in fixed

tissue sections has been successfully achieved. Oncogene proteins have been found in Harvey virus-induced sarcomas, and progress is being made towards the demonstration of these proteins in chemically induced tumors in early stages of development, which is expected to yield important information on the role of activated oncogenes in different stages of the development of chemically-induced neoplasms.

There is evidence from studies of mouse thymomas that oncogene activation may be carcinogen-specific. However, for most rodent tumor models and human tumors, the possible role of specific carcinogenic etiology in oncogene activation has not been explored. In order to conduct such a comparative study, DNAs from rat nasal and mouse skin carcinomas and fibrosarcomas induced by the alkylating agents methylmethane sulfonate (MMS), beta-propiolactone (BPL) and dimethyl-carbamylchloride (DMCC) were tested for their ability to transform NIH 3T3 cells by DNA transfection. The three compounds produce different DNA adduct patterns when reacted to DNA *in vitro*, but were shown to induce tumors of the same histological types in the rat nasal cavity or mouse skin. Each of 8 MMS-induced rat nasal carcinomas and 2 of 5 BPL-induced mouse skin tumors were shown to be positive in the transfection assay, while all of 4 fibrosarcomas and 6 carcinomas induced by DMCC were shown to be negative. The transformed phenotype of the positive transfectants was confirmed by their anchorage-independent growth, tumorigenicity in nude mice, and secondary transfection. The transfectants from MMS-induced tumor DNAs were shown to not contain restriction fragments homologous to rat H-, K- or N-ras oncogenes, although exogenous (rat) tumor-derived DNA sequences were detected in transfected genomes by Southern blot analysis. In contrast, a BPL-induced mouse skin tumor showed evidence of containing activated H-ras. These results suggest a specificity among causal chemical carcinogens for activation of transforming genes in experimental tumors. Further study of animal tumors induced by carcinogens with well-defined biochemical reactivities will be required to elucidate the specific relationships between the biochemical and molecular mechanisms of carcinogenesis.

The mechanism by which chemical carcinogens may activate proto-oncogenes was explored by analyzing the distribution of carcinogen adducts on different parts of the genome. BP adducts, formed *in vivo* in hamster liver cells, were preferentially located in DNAase I hypersensitive regions of the genome and were rapidly removed by repair processes, while persisting adducts remained in other parts of the genome. In target hamster liver cells, the H-ras proto-oncogene was found to be present in a transcriptionally active form.

Epidermal differentiation is associated with the modulation of expression of specific genes, including a gene coding for a precursor protein for cornified envelope assembly and a protease inhibitor gene. The expression of these genes is also modified in epidermal tumors. The ras oncogene is highly correlated to the initiated phenotype in epidermis. Introduction of an activated ras gene into normal keratinocytes converts them into papilloma cells. Chemically-induced papillomas yield an activated ras oncogene with a mutation at codon 61. The activation of a ras oncogene in papilloma cells is associated with the expression of a unique keratin protein which is normally expressed only in simple epithelia. Papilloma cells and initiated cells are resistant to the differentiation-inducing effects of phorbol ester tumor promoters and to the cytotoxic effects of benzoyl peroxide. Since phorbol esters induce differenti-

ation in normal cells, papilloma cells can be selected among an excess of normal cells in culture by their ability to continue to proliferate in culture medium containing phorbol esters. In vivo, several classes of benign tumors can be induced by initiation and promotion. Tumors can be subdivided into those with a high risk for malignant progression and those with a very low risk for malignant progression. Future studies are designed to determine whether a specific set of genes, which predetermine the biologic potential of the tumor cell, is activated during initiation.

There is a need for additional experimental models to study the series of steps initiated by damage to DNA which leads to the genomic changes that result in malignancy. A guinea pig model, with discrete preneoplastic stages, provides an ideal opportunity for studying multistage oncogene-induced neoplasia. Five independent tumorigenic guinea pig cell lines initiated by diverse chemical carcinogens (aromatic aryl hydrocarbons or alkylating agents) contain activated oncogenes that transform NIH 3T3 cells. Oncogene activation occurred at a late stage of carcinogenesis closely associated with acquisition of tumorigenicity. Sequence analysis of the cloned oncogene of one line shows that it is an N-ras gene activated by an AT to TA transversion at the third position of codon 61. This results in the insertion of histidine instead of glutamine. The other four lines contain the same activated N-ras gene with the identical mutation. These results suggest that the mutational event was independent of the mutagenic activity of the initiating carcinogen. The activation of ras genes in in vitro models usually occurs in an early step and the specific activating mutation is generally related to the initiating carcinogen. The contrasting results with the guinea pig model demonstrates that ras activation need not occur at a specific interval in the carcinogenesis process.

Position 12 is one of the important mutation sites in the ras family of viral oncogenic proteins, when comparison is made to their analogous cellular non-transforming and transforming p21 proteins in terms of physicochemical properties of these molecules. Both the glycine-containing and the valine-containing N-terminal 34-residue peptide segments encompassing position 12 of the ras protein have been synthesized. To date, the glycine-containing peptide has been characterized using circular dichroism or P-31 nuclear magnetic resonance spectroscopy and equilibrium dialysis methods. The results indicate that both GTP and ATP form complexes with the gly-peptide indiscriminately and that a longer segment, possibly the intact 189-residue p21 protein chain, is required for the assumption of the proper tertiary structure such that binding occurs exclusively with GTP and GDP. Furthermore, circular dichroism experiments indicate that addition of the nucleotide to the gly-peptide induces conformational changes in the peptide. When the amino acid at position 12 is valine, such conformational changes may be impeded. This hypothesis is being tested.

Studies on Gene Expression and Growth Control of Normal and Tumor Cells

Studies with keratinocytes have indicated that initiation of carcinogenesis is associated with a change in normal differentiation. The regulation of specific differentiation products is being explored in order to understand this association at the molecular level. cDNA clones corresponding to the major keratins expressed in mouse epidermis have been isolated and characterized. Using a

combination of *in situ* hybridization with RNA probes, which are specific for individual keratin mRNAs, and indirect immunofluorescence with monospecific antisera which were elicited with synthetic peptides corresponding to unique sequences within each keratin subunit, it is possible to show that these keratin genes belong to at least three subsets: those expressed predominantly in the proliferating basal layer of the epidermis, those expressed predominantly in the differentiated suprabasal layers of the epidermis, and those expressed only under hyperproliferative conditions such as in benign and malignant epidermal tumors, hyperplasia induced by the tumor promoter TPA and squamous metaplasia induced by various means in hamster trachea. Genes representing each subset have been isolated and sequenced. Various strategies have been employed to identify sequences regulating the expression of these genes, including vector constructs using different regions of the genomic clones to drive expression of the neomycin-resistance gene and the chloramphenicol acetyl transferase gene and the production of transgenic mice containing genomic fragments encoding human keratins. The *in situ* hybridization technique has been used to confirm previous results obtained with monospecific antisera which demonstrates that malignant epidermal tumors could be distinguished from benign tumors due to their failure to express the differentiation-associated keratins. The *in situ* hybridization technique has also been used to study the expression of human keratin genes in epidermal tumors and other skin disorders. In addition, this technique has been used to localize transcripts of other genes that are differentially expressed in the epidermis.

Vitamin A is essential for the normal growth and differentiation of epithelial cells and has been shown to serve as a regulator of the dolichyl phosphate-mediated pathway of protein glycosylation. Vitamin A deficiency causes a marked (up to 95%) decrease in the incorporation of 2-[³H]mannose into glycoproteins *in vivo*. The entire pathway from mannose to mannose-phosphate, guanosine diphosphomannose, dolichyl phosphate mannose, lipid-linked oligosaccharides and glycoproteins was investigated to determine the steps controlled by the vitamin A status in hamster liver. The primary effect was shown to be on the biosynthesis of GDP-mannose. Since enzyme activity responsible for GDP-mannose synthesis from GTP and mannose phosphate was not influenced by vitamin A deficiency and mannose phosphate was found to accumulate, it appears that lower concentrations of GTP are responsible for the observed effect of vitamin A deficiency. These findings were confirmed in organ culture of hamster trachea, where deficiency caused an accumulation of mannose phosphate and a decrease in GDP-mannose labeling, using 2-[³H]mannose as the precursor. Serum retinol binding protein (RBP) is responsible for the transport of retinol from the liver to other vitamin A-requiring target cells such as the epidermis. Several studies suggest that RBP is recognized by target cells through a specific cell surface receptor. Primary mouse epidermal cells were used as a model system to more clearly delineate the steps involved in the delivery of retinol from RBP to target cells. RBP was purified from rat serum, loaded with [³H]retinol, and purified the [³H]retinol-RBP complex by affinity chromatography on human prealbumin Sepharose. Epidermal cells incubated with [³H]retinol-RBP complex accumulate cell-associated radioactivity in a time-dependent manner. The uptake of [³H]retinol from the [³H]retinol-RBP complex was inhibited by unlabeled holo-RBP with an apparent K_m of 2-4 μM , the concentration at which RBP is normally found in serum. [³H]-Retinol uptake from RBP was not influenced by inhibitors of receptor-mediated endocytosis and using ¹²⁵I-labeled RBP no evidence

of direct binding or internalization of RBP was found. Homogenization and centrifugation of cells following delivery of [^3H]retinol from RBP determined that 80% of the cell-associated radioactivity was membrane bound. These results are consistent with retinol being delivered from RBP to epidermal cells via a transitory interaction with a cell surface receptor.

Regulatory signals involved in the control of mouse mammary tumor virus (MMTV) transcription by glucocorticoids continue to be investigated using cell lines conditionally transformed by $v\text{-ras}^{\text{H}}$ oncogene. In those cells, the $v\text{-ras}^{\text{H}}$ gene is driven from the MMTV promoter; as a result, levels of the p21 gene product are subject to regulation by glucocorticoids. Two rare transfectants have been characterized in which the level of p21 protein is sufficiently low minus hormone that the cells revert to normal phenotype in the absence of hormone. Studies are underway to identify potential changes in gene expression during this phenotype switch utilizing high-resolution twodimensional gel technology. The availability of a system for conditional expression of the transformed phenotype has stimulated considerable interest, and many groups have applied this approach in the study of different oncogenes, as well as other cellular functions.

The regulation of gene expression in eukaryotic cells by estrogens and other steroid hormones involves the interaction of specific intracellular receptor proteins with the genome, resulting in the activation of selected sets of responsive genes. A complementary DNA clone (cDNA) containing the entire translated portion of the messenger RNA for the estrogen receptor from human breast cancer cells was sequenced. This cDNA provided a functional protein (gene product) when introduced into Chinese hamster ovary cells. Amino acid sequence comparisons revealed significant regional homology among the human estrogen receptor, the human glucocorticoid receptor, and the putative $v\text{-erb-A}$ oncogene product. This suggests that steroid receptor genes and the avian erythroblastosis viral oncogene are derived from a common primordial gene. This oncogene is unusual in that it shares the genome of the avian erythroblastosis with another oncogene, the $v\text{-erb-B}$. The $v\text{-erb-B}$ oncogene was transduced from the gene that encodes the cell surface receptor for epidermal growth factor. This homology of estrogen receptor to an oncogene links the hormone action with carcinogenesis. The cloning of the cDNA and natural gene for human breast cancer cell estrogen receptor will enable investigators to obtain large quantities of human receptor for detailed analysis of structure, composition and function. In addition, introduction of the cloned cDNA into hetero-specific prokaryotic and eukaryotic cells will permit a study of the regulation and organization of these genes.

The control of HLA-DR expression was studied in cell lines derived from patients with adult T-cell leukemia and childhood acute lymphocytic leukemia (ALL). The former express HLA-DR antigens and are infected with HTLV-I, while the latter are not infected with HTLV-I and express no HLA-DR antigens. Phorbol esters induce expression of HLA-DR antigens and demethylation of HLA-DR genes in all ALL-derived T-cell lines. In short-term culture of fresh lymphocytes, adult T-cell surface markers occur at the same time as expression of viral proteins. The HLA-DR genes were found to be methylated to the same extent as the fresh adult T-cell leukemias. The results suggest that the HTLV-I retrovirus induces or controls HLA-DR expression at a post-transcriptional level.

Studies on Carcinogen Metabolism and Interaction with DNA

The earliest events in the multistage process of chemical carcinogenesis are considered to include (1) exposure to the carcinogen; (2) transport of the carcinogen to the target cell; (3) activation to its ultimate carcinogenic metabolite, if the agent is a procarcinogen; and (4) DNA damage leading to an inherited change. Studies on the metabolism of chemical carcinogens are important because (1) many environmental carcinogens require metabolic activation to exert their oncogenic effects; (2) the metabolic balance between carcinogen activation and deactivation may, in part, determine a person's oncogenic susceptibility; and (3) knowledge of the comparative metabolism of chemical carcinogens among animal species will aid efforts to extrapolate data on carcinogenesis from experimental animals to humans.

Xenobiotics such as drugs and carcinogens, as well as endobiotics such as steroids and fatty acids, are metabolized by the mixed-function oxidase systems. Cytochrome P-450 is the key component of mixed function oxidases and the type and quantity of specific forms of cytochrome P-450 determine the disposition of a particular substrate.

Several forms of P-450 were purified from rat liver and kidney, and rabbit liver microsomes. These purified P-450s were characterized catalytically, structurally and spectrally, and classified on the basis of immunological cross-reactivity. Several forms of P-450 were used to study the conformation of the protein surface, using monoclonal antibodies (MAbs) to specific cytochromes P-450. MAbs were also used for immunopurification of P-450 with catalytic activity, for making a P-450-bioreactor and for evaluating epitope topography of the microsomal membrane. Particular efforts have focused on the enzyme P-450j which has a high activity toward the potent carcinogen N-nitrosodimethylamine. The demethylation reaction carried out by this P-450 leads to highly reactive intermediates that can bind and mutate DNA. A monoclonal antibody against rat P-450j was used to isolate rat and human P-450j cDNA clones. These cDNAs were sequenced and their nucleotide and deduced amino acid sequences were 78 and 80% similar, respectively. The rat P-450j monoclonal antibody and cDNA clones were utilized as probes to determine the mechanism whereby P-450j is regulated by ethanol, acetone, and the alcohol dehydrogenase inhibitors, 7-methylpyrazole and pyrazole. A unique translational control of P-450j by these compounds was uncovered. The fourfold elevation in P-450j protein in rat microsomes by these agents is not accompanied by an increase in its mRNA. In contrast to this post-transcriptional regulation, P-450j is increased during the first few days after birth through a massive increase in gene transcription rate.

Infectious recombinant vaccinia viruses have been constructed containing the full length cDNAs of P1-450 and P3-450. Human and mouse cells infected with the recombinant viruses showed high level expression of the authentic proteins as detected by immunoblotting. The expressed proteins are enzymatically active and exhibit distinguishable substrate specificities.

The metabolic processing of chemical carcinogens by human tissues has been studied. Two distinct phenotypes, slow and fast metabolizers, were observed for both metabolic activation and detoxification of the model carcinogen,

2-acetylaminofluorene (AAF), in human liver microsomes from 28 individuals. Individuals who were fast activators of the carcinogen were, in most cases, also fast detoxifiers of the chemical. However, different phenotype patterns exist suggesting that fast activators of a toxin need not also be phenotyped as fast detoxifiers. The metabolic activation of the amino acid pyrolysate, Trp-P-2, to a mutagen by the same human liver samples was also polymorphic and there was a highly significant correlation between the N-hydroxylation of AAF and the mutagenicity of Trp-P-2. Studies in this area may help to understand whether certain individuals are predisposed to a higher rate of chemically-induced cancer.

Whether or not they require metabolic activation, most chemical carcinogens bind covalently to DNA and their reactions are thought to be critical in the initiation of carcinogenesis. Antibodies specific for carcinogen-DNA adducts have probed the nature, extent, and consequences of *in vitro* and *in vivo* DNA modification. DNAs substituted with 2-acetylaminofluorene (AAF) and *cis*-diamminedichloroplatinum II (*cis*-DDP) were analyzed by quantitative immunoassays able to detect about 100 adducts per cell, and by immunohistochemical procedures developed to localize adducts *in situ*. In hepatic DNA of rats fed a carcinogenic dose of AAF for 4 weeks, adduct accumulation reached a plateau in 2-3 weeks. Since adduct removal was biphasic during 4 subsequent weeks on control diet, a computer-derived pharmacokinetic model proposed that adducts are formed into two genomic compartments, one from which they are removed rapidly and another from which they are removed slowly. Studies initiated to identify these two compartments involve adduct analysis in different liver cell types and different chromatin regions including the nuclear matrix. Immunohistochemical localization of AAF-DNA adducts in livers of rats fed AAF was shown by fluorescence and microfluorometry to be concentrated primarily in periportal regions. In addition, there were no adducts detectable by immunofluorescence in preneoplastic foci induced by several different protocols. Nucleated peripheral blood cell DNA was obtained from cancer patients at multiple times during courses of *cis*-DDP therapy, and a total of 254 samples were analyzed. Of these, 23 untreated control samples were negative, and 43% of the 231 samples from patients receiving *cis*-DDP were positive. Adduct accumulation, in positive patients, occurred as a function of total cumulative dose, suggesting relatively slow adduct removal. Disease response data on 55 patients indicated that individuals with high adduct levels have a high rate of complete response to therapy. Parallel experiments in animal models have demonstrated that the same adduct forms in kidney, gonads, and tumors of rats and mice in direct relation to dose.

Benzo[a]pyrene (BP) is a ubiquitous carcinogen found in tobacco smoke, burning fossil fuels, and our diet. Formation of BP diol epoxide (BPDE)DNA adducts due to human exposure is most likely to be at very low levels that are beyond the sensitivity of routine radioimmunoassay and chromatographic analyses. Thus, ultrasensitive enzymatic radioimmunoassay (USERIA), enzyme-linked immunosorbent assay (ELISA), and synchronous scanning fluorimetry have been employed to detect and quantitate BPDE-DNA adducts in humans at high cancer risk due, in part, to BP exposure. DNA isolated from white blood cells of asphalt workers (roofers) and foundry workers and DNA from lung tissue, bronchial washings,

and alveolar macrophages of lung cancer patients and smokers are being investigated. Putative BPDE-DNA adducts have been detected in several of the high-risk individuals. Antibodies to BPDE-DNA adducts were also found in sera from those workers. These data suggest that the activation of BP to its ultimate carcinogenic form, as well as formation of adducts with DNA, occurs in humans. Preliminary evidence indicates that other polycyclic aromatic hydrocarbons, e.g. chrysene- and benzo(a)anthracene-DNA adducts, may also evoke an immune response in humans.

A method known as the ^{32}P -postlabeling technique has recently been developed for detecting the in vivo formation of carcinogen-DNA adducts in which the use of radiolabeled test compounds is not required. The method involves the reaction of DNA with chemicals in vitro or in vivo and the purification and enzymatic digestion of DNA to deoxyribonucleoside 3'-monophosphates. These are converted to ^{32}P -labeled deoxyribonucleoside 3',5'-biphosphates after incubation with (gamma- ^{32}P) ATP and T4 polynucleotide kinase. The ^{32}P -labeled digests are then fingerprinted by using reversed-phase liquid chromatography and anion-exchange thin-layer chromatography on polyethyleneimine-cellulose followed by detection by autoradiography and quantitation by scintillation counting. The test conditions have been further refined by chromatographically removing normal DNA nucleotides prior to ^{32}P -labeling. This has led to a greatly increased overall sensitivity of adduct detection and more accurate quantitation of adducts. This method has been applied to answer questions about the tissue specificity of carcinogen adduct formation, the persistence of DNA adducts in cells, and to detect adducts in cells of humans exposed to carcinogenic chemicals. In one such study the question of DNA adduct formation in diethylstilbestrol (DES)-induced renal carcinogenesis in vivo was investigated. There is a current controversy concerning the mechanism of carcinogenesis by DES. DES and other synthetic and natural hormones have been shown to act as promoters of tumorigenesis and have thus been viewed as examples of epigenetic carcinogens, compounds that can induce cancer without direct interaction with genetic material. This view is supported by the failure of DES or its derivatives to cause mutations in the Ames assay. However, DES induces sister chromatid exchanges, unscheduled DNA synthesis and aneuploidy in Syrian hamster embryo cells, suggesting that DES can damage cellular macromolecules. The ^{32}P -postlabeling assay detected the presence of covalently modified DNA nucleotides in the kidneys after chronic DES treatment, but not in kidneys of untreated hamsters or in liver or tumor tissue of DES-treated animals. At least three different adduct spots were detected. This is the first demonstration of the ability of an estrogen to give rise to covalent DNA modification in vivo specifically in the target organ of carcinogenesis. The structures of the modified nucleotides have not yet been determined. Recent observations, however, show that the DNA adducts observed do not contain estrogen-derived moieties. The above results indicate that DES can be classified as a complete carcinogen and has led to the conclusion that estrogens such as DES induce the binding of some endogenous compound(s) to target tissue DNA.

In another application of the ^{32}P -postlabeling method, the presence of covalent DNA adducts in human full-term placental samples was investigated. Maternal blood and cord blood samples and placentas were obtained at delivery from volunteers. Blood specimens were analyzed for three biochemical markers of smoking exposure--cotinine, thiocyanate, and carboxyhemoglobin--and smoking

data was collected from each volunteer. DNA was also analyzed for adducts by ELISA with antibodies to DNA modified by antibenzo(a)pyrene diol epoxide (BPDE-I). The ELISA detected a small but statistically significant increase in adduct levels in placental specimens of smokers. The post-labeling assay detected up to five modified nucleotides, one of which was strongly related to maternal smoking during pregnancy. This adduct was shown to be present in placental tissue from 16 to 17 smokers, but in only 3 of 14 nonsmokers. One adduct was presumed to be a derivative of an aromatic carcinogen, but when co-chromatographed with test DNA preparations containing DNA adducts from various aromatic carcinogens known to be present in cigarette smoke, none co-chromatographed. Efforts are continuing to identify this adduct. The data show the association of cigarette smoking with covalent damage to human DNA in vivo and demonstrate that this analytical approach should contribute to a definition of chemical components of cigarette smoke as well as other environmental exposures that most severely damage human DNA.

Gamma-glutamyl transpeptidase (GGT) is a membrane-bound glycoprotein enzyme of unknown structure that is involved in the degradative metabolism of glutathione and the uptake of some amino acids. In rodents, GGT activity is especially high in fetal liver and in adult kidney but not in adult liver. The low liver enzyme activity is inducible by certain drugs and carcinogens such as azo dyes. For example, elevated levels of GGT are found in most hepatomas. This enzyme is thus expected to be a marker of neoplastic transformation, especially in hepatoma cells. It is also known that there are structural differences between the enzymes found in tumorigenic tissue and the corresponding normal tissues. The active heterodimeric enzyme from adult rat livers has been purified to homogeneity. A fast preparative HPLC method was developed to separate the two enzymic subunits under acidic conditions. 2D-PAGE was found to resolve the active enzyme into at least 18 components. Seven components with apparent molecular weight of 51-53,000 comprised the heavy subunit. Immunoblot analysis of 2D-PAGE showed that all of these components are immunoreactive with a mixture of the two antibodies generated separately against the light and heavy subunits, demonstrating that they are all valid constituents of the enzyme complex. N-Terminal amino acid sequencing of the separated subunits of the enzyme yielded, for the first time, sequence information for the first 32 residues of the heavy chain and for 36 residues of the light subunit. The sequence information allowed the construction of oligonucleotide probes for cloning of the GGT gene. Whereas the 2D-PAGE results indicate considerable heterogeneity for the active enzymes, the sequencing information, at least to the extent determined, confirms earlier observations that the heterogeneity does not reside in the amino acid sequence of the enzyme, but rather in the attached carbohydrate chains.

Studies on DNA Repair and Chromatin Structure

In the past few years evidence has accumulated that DNA repair is far more complex than anticipated and that the classical nucleotide excision pathway is integrated with other equally important mechanisms to maintain the integrity

of DNA. Previous investigations studying the preferential formation of DNA-protein cross-links and single strand DNA breaks in areas of the genome containing transcriptionally active genes found that after x-irradiation two nonhistone proteins which are minor components of the nuclear matrix become covalently bound to the DNA. During the repair process the protein linked to DNA in regions of transcriptionally active areas is removed, following which other areas of the genome are bound. Recent studies have shown that there are about 6000 DNA-protein cross-links in the normal cell and that for each Gy of x-irradiation an additional 150 cross-links are induced. These x-ray-induced cross-links are preferentially formed between actively transcribing DNA and protein of the nuclear matrix. This was demonstrated by showing that the mouse globin gene is hypersensitive to formation of radiation-induced DNA-protein cross-links only when it is in the matrix-associated transcribing mode of stimulated erythroleukemia cells but not in the unstimulated mode of erythroleukemia cells or in fibroblasts. Similar experiments showed that a heat-shock gene also was hypersensitive to DNase I digestion in the transcribing mode but not sensitive to digestion in the latent, "turned off," mode. These results underscore the complexity of cellular responses and show that the DNA target genome is not equally sensitive to x-ray throughout its length. Further, sensitivities to radiation not only change during the cell cycle, but they change according to whether or not the gene is activated or suppressed.

The transfection technique of DNA information transfer has become a powerful method of introducing specific genes into mammalian cells after appropriate manipulation by recombinant DNA methods. However, most human cells have proven refractory to integration of the DNA into a stable state of expression. Last year it had been discovered that UV irradiation of chimeric plasmids such as pSV2-gpt results in a remarkable enhancement in the yield of gpt⁺ transformants when the plasmids are transfected into human cells by the calcium phosphate technique. Further, only "bulky" lesions, including pyrimidine dimers and psoralen adducts, cause this enhancement which can be as much as 20-fold. This has been extended to show that pyrimidine dimers in the DNA sequences flanking the transforming genes in shuttle vectors are responsible for the marked transformation enhancement of human cells. Further, the pyrimidine dimers are the predominant lesion involved and the average number of copies of the gene integrated into the genome of the transformant is not affected by the damage. Techniques to measure specific DNA damage and its repair in a defined nucleotide sequence at the single-copy level in the mammalian genome have also been developed. Using these techniques it has been discovered that the essential dihydrofolate reductase gene is proficiently repaired in normal human fibroblasts and in Chinese hamster ovary cells (CHO) with overall low repair ability, but not in cells from individuals with the hereditary disease xeroderma pigmentosum (complement group C) which have low repair levels like CHO cells. These extraordinary experiments show that cellular survival correlates with efficient repair in an essential gene rather than with overall repair levels and resolves a longstanding anomaly concerning the relationship between DNA repair capacity and survival in rodent and human cells in culture. In addition to the usefulness of this discovery to manipulate DNA in human cells in culture, these results further emphasize the need to better understand the relationships which exist between DNA repair, replication, integration, and expression and to recognize the interdependence of these processes and their response to UV radiation.

New assays utilizing plasmids as tools to measure DNA repair and mutagenesis at the molecular level have been developed and applied to studies of patients with xeroderma pigmentosum (XP) and with the dysplastic nevus syndrome (DNS) of hereditary cutaneous melanoma. In the XP cells, one pyrimidine dimer blocks expression of a transfected gene. Non-dimer photoproducts also block expression in XP cells. In normal cells, UV pretreatment of a shuttle vector plasmid, pZ189, resulted in appearance of transitions and transversions. Survival of UV-treated pZ189 was reduced in the XP cells and a restricted mutagenic spectrum was found. In pZ189 replicated in XP cells 93% of the base substitution mutations were GC to AT transitions ($p < 0.0009$). The major UV photoproduct, the thymine dimer, was only weakly mutagenic. Cultured lymphoblastoid cells from familial DNS patients were hypermutable to UV. In a retrospective study of more than 700 XP patients, it was shown that they have a greater than 100-fold increased risk of developing basal cell or squamous cell carcinoma or melanomas of the skin. The reported median age of first skin cancer was less than 10 years, a 50-year reduction in comparison to the United States general population.

The role of chromosomal proteins in maintaining the structure and regulating the function of chromatin and chromosomes is being studied. Specific antibodies were used to construct immunoaffinity columns for fractionating chromatin. Studies on the exchange of proteins during immunofractionation of chromatin revealed that at low ionic strength there was negligible exchange of proteins between nucleosomes. Nucleosomes enriched in HMG-17 or H10 have been isolated, by immunoaffinity chromatography, and the DNA examined with a variety of genetic probes. The results indicate that chromatin fragments containing DNA sequences with an open reading frame are enriched in HMG-17, while nucleosomes containing DNA sequences coding for inducible proteins are depleted of H10. cDNA coding for human chromosomal protein HMG-17 was cloned and sequenced and used to probe the genomic organization of the gene. In the human genome, there are over 50 gene equivalents for this cDNA, suggesting that the protein is encoded by a multigene family. Southern analysis of the DNA from several transformed human cell lines failed to detect any restriction fragment polymorphism in this gene. The cDNA has some unusual characteristics: only 25% of the transcript is translated; the 5' untranslated region is extremely rich in GC residues, while the 3' untranslated region is very rich in AT residues.

The chromatin structure of genes coding for P-450 enzymes is being investigated. Changes in the chromatin structure upon gene activation is examined by comparing the chromatin structure of these genes in nuclei purified from either normal or carcinogen-treated rats. Micrococcal nuclease digestion revealed that, in the liver, genes coding for the 3-methylcholanthrene-inducible P-450c, P-450d, and P-450m enzymes are not organized in the typical 200 base pair nucleosomal conformation. DNase I hypersensitive sites were mapped in each of these genes. These sites were not observed in nuclei isolated from the thymus. These results suggest that in the liver the chromatin structure is altered to allow tissue-specific gene expression. Gene induction by carcinogen treatment is associated with a rearrangement of the nuclear location of the P-450c and P-450d genes and with a change in one of the DNase I hypersensitive sites present in the P-450c gene. The results suggest that gene induction is associated with detectable changes in the chromatin structure of these genes.

Studies on Growth Factors and Lymphokines

The isolation, characterization and biological role of transforming polypeptide growth factors (TGFs) continue to be a major focus of effort. There is now a solid base of data supporting a bifunctional role of TGF- β in the regulation of cellular proliferation, cellular differentiation, and cell function. For example, TGF- β synergizes with EGF and with PDGF to stimulate anchorage-independent growth of certain cells, while it blocks the mitogenic effects of EGF and PDGF on growth of the same cells in monolayer culture. TGF- β also has profound effects on lymphocytes and can block the mitogenic effects of interleukin-2 on T-cells and can also block secretion of immunoglobulins by B-cells. TGF- β has recently been found to stimulate formation of extracellular matrix by cells of mesenchymal origin. Cells of the immune system and osteoblasts have been found to secrete relatively large amounts of TGF- β , suggesting that fibrosis that accompanies chronic inflammation, as well as matrix formation by bone-forming cells, may be dependent on TGF- β . Investigations of embryonic development have shown unusually high expression of TGF- β , mRNA throughout embryogenesis; this, too, may be correlated with a requirement for matrix synthesis.

The complete rat TGF- α gene has been synthesized and expressed in a retrovirus vector. NRK cells have been infected with recombinant viruses carrying this TGF- α gene, and these cells have the transformed phenotype in the presence of TGF- β . Studies have been performed on the levels of TGF- β in monocytes, both before and after their activation to macrophages. Activation causes a definite increase in the production of TGF- β mRNA, as well as an increase in secreted protein. These studies are of major importance with respect to the role of the macrophage as a key mediator in inflammatory reactions. Polyclonal antibodies have been raised against human TGF- β . Immunoglobulin fractions have been prepared from this serum by affinity chromatography. These antibodies effectively block binding of TGF- β on NRK cells. This antiserum was used to demonstrate that TGF- β is the principal component in serum that induces the terminal differentiation of human bronchial epithelial cells.

Lymphokines are now recognized as important regulators of cell growth and differentiation. As such they have the potential to interact with and to modify many stages in the development of neoplasia. One lymphokine, leukoregulin, has anticarcinogenic and antitumorigenic properties. The uniqueness of this hormone, moreover, is that it acts directly on the target cell in contrast to stimulating the activity of cytotoxic effector cells as do lymphokines like interferon and interleukin-2. Interaction of leukoregulin with the target cell is followed by alterations in cell surface conformation, plasma membrane integrity, cell surface glycoprotein expression, and DNA synthesis. The ability of leukoregulin to effect changes in both cell metabolism and structure makes this "biological" a valuable probe for investigating the extragenetic control of carcinogenesis and the neoplastic growth of transformed cells.

Interaction of leukoregulin with the target cell initiates a series of events commencing with an immediate, continuous opening and closing of plasma membrane monovalent ion channels, a marked increase within a minute or so in intracellular ionic calcium and several minutes later in a progressive increase in plasma membrane permeability followed by increased sensitivity of the affected target

cell DNA synthesis. Leukoregulin's alteration of calcium flux and membrane permeability, without causing cytolysis, demonstrates that neither event alone or in combination is necessarily cytolethal. This is consistent with leukoregulin serving as an immunologic hormone whose function is to condition abnormal cells for eventual elimination by the host. Modulation of cell surface conformation, membrane fluidity, and permeability to the same degree present in natural lymphocyte-mediated cytotoxicity reactions, moreover, signifies that interleukin through transmembrane signaling has the potential to control the specificity of membrane cytotoxicity by decreasing the proliferative rate of the target cell and increasing the vulnerability of its plasma membrane to additional cytoregulatory agents. Changes in calcium flux may also be important in the anticarcinogenic action of leukoregulin, but diverge from the molecular pathway leading to increased membrane permeability since leukoregulin does not decrease membrane integrity or inhibit DNA synthesis in nontransformed cells serving as targets for carcinogenesis. Leukoregulin is the only lymphokine known to increase the susceptibility of pre-neoplastic and neoplastic cells to destruction by natural killer lymphocyte cytotoxicity and to reversibly inhibit DNA synthesis in the cells. The specificity of the latter two actions is extremely high and is mediated through leukoregulin-induced destabilizations of the cell surface and in the integrity of the target cell plasma membrane. In this manner leukoregulin may function as one of the earliest intrinsic mediators in natural lymphocyte cytotoxicity by controlling the specificity and direction of the immunoregulatory action directly at the surface of the pre-neoplastic or neoplastic target cell. The specificity and direct target cell actions of the lymphokine make leukoregulin a valuable new probe for study of the extragenetic prevention and control of carcinogenesis and as a potential agent for the control of tumor and other abnormal cell growth.

Epidemiology and Biostatistics

Continued emphasis was given this year to case-control and cohort studies aimed at evaluating key hypotheses in cancer etiology. Case-control studies of selected cancers have been undertaken when high-risk communities are identified on the cancer maps or when major testable hypotheses and special resources become available. Whenever possible, laboratory procedures are incorporated into the epidemiologic studies to better clarify exposures, pre-clinical responses, and mechanisms of carcinogenesis. Although descriptive studies were less prominent this year, systematic surveys were conducted to elucidate time trends in cancer incidence and mortality. An updated atlas was prepared to illustrate the geographic patterns of cancer mortality by state economic area in the 1970s, and comparison was made to patterns for the two earlier decades.

Biochemical Epidemiology

Multidisciplinary projects combining epidemiologic and experimental approaches have been emphasized to evaluate the influence of viruses, dietary and metabolic factors, host susceptibility, air and water pollutants, and a wide variety of other possible cancer risk factors. Laboratory measurements of these factors allow investigators to assess past exposures and subclinical or preclinical response to initiators, promoters, and

inhibitors of carcinogenesis and to evaluate host-environmental interactions. The Program is seeking ways to utilize this approach to clarify carcinogenic risks associated with certain micronutrients or environmental agents that can be detected in tissues or body fluids. Opportunities are also being sought to assess specific host factors that influence susceptibility to cancer, including endocrine function, immunocompetence, and genetic markers including oncogenes. Of special interest are techniques to detect and quantify exposure to particular carcinogens or their metabolites in vivo through chemical analyses, mutagenesis assays, or immunologic detection techniques. Ways of measuring the interaction of certain agents with cellular target molecules include adduct formation with proteins and nucleic acids, excretion levels of excised adducts, and markers of altered gene expression. Collaborative investigations are underway using these experimental tools in studies of lung cancer and to evaluate the role of fecal mutagens in the development of colorectal cancer. Collaboration with other intramural laboratories are ongoing in viral carcinogenesis, especially to evaluate the role of retroviruses and papillomaviruses in human cancer. Other projects are investigating the role of human leukocyte antigens (HLA), micronutrients (vitamins and trace metals), and endogenous hormones as cancer risk factors. A request for applications (RFA) was issued in the area of biochemical epidemiology to enhance the development, validation and application of laboratory procedures in detecting human environmental exposures that might affect cancer risk. This initiative is being jointly supported by NIOSH, EPA, and the National Institute of Environmental Health Sciences.

Diet, Nutrition and Cancer

Research in this area was further intensified as evidence accumulates to suggest that dietary factors contribute to a large though uncertain fraction of human cancer. A number of studies of diet and cancer have been conducted in high-risk areas of the country. In a case-control study of lung cancer among white men in New Jersey, carotenoid intake was protective, with those in the lowest quartile of consumption having 1.3 times the risk of those in the highest quartile after adjusting for smoking. No increase in risk was associated with low consumption of retinol or total vitamin A. Intake of vegetables, especially dark green and dark yellow-orange vegetables, showed even stronger inverse associations than the carotenoid index, possibly because of the high content of beta-carotene in those food groups. The protective effect of vegetables was limited to current and recent cigarette smokers, with the smoking-adjusted relative risk for low vegetable consumers reaching 1.7 times the risk for high consumers. This suggests inhibition of a late-stage event of carcinogenesis. It is noteworthy that a case-control study of esophageal cancer among black men in Washington, D.C. implicated a generalized deficiency state and alcohol consumption as the major predictors of risk in this high-risk population, while a study of oral and pharyngeal cancer among southern women suggested that vegetable and fruit intake was protective, possibly because of micronutrients such as beta-carotene or vitamin C. Recent case-control studies of pancreatic and stomach cancer in Louisiana indicated that fruit intake alone is protective; alcohol and coffee intake were unrelated to the risk of pancreatic cancer. In all these studies, people in the highest quartile of consumption of a particular food group were able to attain cancer risks 50-70% or less, compared to those in the lowest quartile, without resorting to drastic

modifications of diet or food or vitamin supplements. Opportunities to study nutritional hypotheses in high-risk areas exist in other countries, such as China, where collaborative case-control and intervention studies are underway.

Infectious agents: Increasing attention was devoted to investigating the role of human retroviruses as a cause of cancer. Epidemiologic studies have clarified the spectrum of T-cell malignancies linked to human T-cell lymphotropic virus type 1 (HTLV-I) and the long latency between infection and leukemia/lymphoma. Both virus infection and T-cell malignancy were found to cluster in certain areas of southern Japan, black populations of the southern United States, the Caribbean basin, some areas of South America, and equatorial Africa. A cohort of Japanese-American men who were born or whose parents were born in viral endemic areas of Japan showed very high rates of HTLV-I antibodies, while a matched comparison group from a nonendemic area had low rates, thus suggesting transmission of the virus in the household, perhaps at birth. Heterosexual transmission was supported by a link between HTLV-I seropositivity and number of lifetime sexual partners in a study in Panama and by an association with VDRL positivity in Barbados. Homosexual contact was also implicated in Trinidad by a sixfold excess associated with a promiscuous life-style.

The Program has been heavily committed to investigating the epidemic of acquired immunodeficiency syndrome (AIDS), which predisposes to Kaposi's sarcoma and opportunistic infection. A series of five cohorts followed since the earliest days of the epidemic have provided a data base for understanding the natural history of the etiologic agent, HTLV-III/LAV, and its pleiotropic effects. Among seropositive individuals, the risk of AIDS was as high as 33 percent over a 3-year period. These studies also documented the modes of transmission via parenteral and sexual routes, but without evidence for casual transmission. Parenteral infection in the health care setting was a rare event, related to percutaneous exposure. HTLV-III/LAV infection appeared to trigger a long process of immunologic impairment, manifested subclinically by a time-dependent ablation of T-helper cells predictive of risk for AIDS. In Africa AIDS appeared to be a new epidemic, and serologic cross-reactivity to HTLV-III/LAV in historic and some contemporary serum collections seemed influenced by malaria infection and perhaps by related, but yet to be characterized, human retroviruses. The classical endemic form of Kaposi's sarcoma in Africa was not related to HTLV-III/LAV and clinical manifestations differed from those of AIDS-related sarcoma. Cytomegalovirus, previously suspected as an etiologic agent of Kaposi's sarcoma, was not linked to African cases on a serologic or molecular basis. Special emphasis is being given to studies which clarify the relation of several viruses to other AIDS-associated tumors (e.g., lymphomas). Also continuing this year were studies to clarify the role of human papillomaviruses in cervical neoplasia and hepatitis-B infection in liver cancer. A serologic follow-up of American veterans who received contaminated yellow fever vaccine in 1942 revealed an infrequency of hepatitis-B carriers, a persistence of viral markers 43 years after infection, and high antibody titers.

Tobacco-Related Cancers

Tobacco habits and lung cancer risks vary geographically in the United States. A case-control study of lung cancer in a high-risk area of southern Louisiana implicated the heavy use, by Cajuns, of local brands and hand-rolled cigarettes containing high-tar levels. A similar study of pancreas cancer in this area also revealed an excess risk among Cajuns that was partly due to smoking habits.

Because of an earlier study linking smokeless tobacco to high rates of oral cancer in the southern United States, the Program has become involved in additional projects to clarify the cancer risks associated with snuff dipping and chewing tobacco. This year Program staff helped organize an NIH Consensus Development Conference on the health implications of using smokeless tobacco and took a lead role in preparing a report to the Surgeon General. Both activities summarized evidence that smokeless tobacco use can cause cancer in humans and is therefore not a safe alternative to cigarette smoking.

Several studies are evaluating the possible association of lung cancer with passive smoking. Data from case-control studies of lung cancer among nonsmoking women in the United States and Japan suggest an increased risk, in proportion to the number of cigarettes their husbands smoked, and indicate the importance of further studies with more quantitative exposure data on various sources of environmental tobacco smoke. Finally, the impact of tobacco on cancer incidence appears to be increasing with a newer understanding about the causes of particular tumors. In a case-control study of patients with invasive cancer of the uterine cervix, a significant dose-related excessive risk was observed for cigarette smoking, after controlling for sexual and other risk factors. Smokers experienced a 50 percent excess risk overall, which rose to twofold among long-term smokers. Thus, the list of cancers associated with tobacco products continues to grow and the need for preventive action is more urgent than ever.

Occupational Studies

As a time-tested means of identifying physical and chemical carcinogens, occupational studies were pursued to assess hazards suspected on the basis of experimental, clinical, and field observations. During the past year a population-based case-control study showed that farmers who used herbicides had a higher risk of developing non-Hodgkin's lymphoma than nonfarmers in the state. Farmers exposed to herbicides, particularly 2,4-D, for more than 20 days each year had six times the risk of developing lymphoma than persons not exposed to herbicides. Among these frequent users, those who mixed or applied the herbicides themselves had eight times the risk of nonusers.

A large-scale study of industrial workers exposed to formaldehyde revealed a slight excess mortality from lung cancer that was not associated with duration or level of exposure. Although based on small numbers, a significant excess of nasopharyngeal cancer was found, mainly following exposure to formaldehyde-containing particulates. A cohort mortality study of members of the American Anatomy Association uncovered a threefold excess of brain cancer and a slight excess of leukemia. Although anatomists may have exposure to formaldehyde, the lack of excesses for brain cancer and leukemia among industrial workers exposed to this substance suggests that some other factor is involved. A case-control study of nasal and paranasal cancers in the Netherlands indicated high risks for adenocarcinoma among persons exposed to wood dusts. The risk was strongest for those newly employed in the 1930s and remained elevated even among those who had discontinued exposure for at least 15 years. The study also uncovered a twofold risk of nasal cancer associated with formaldehyde exposure, particularly for squamous-cell carcinomas, which could not be attributed to smoking or wood dust exposure.

Radiation

Studies were continued to investigate further the relationship between cancer risk and ionizing radiation, especially exposure to high doses, and to improve estimates of risk associated with lower doses. An immediate practical need is for risk estimates on which to base regulatory and other decisions about the use of nuclear and radiological technology in medicine and industry and to assess the value of exposure avoidance as a means of cancer prevention.

In an international study of over 9000 children treated for cancer, the risk of second cancers of the bone and thyroid was strongly associated with high-dose radiation therapy. Radiotherapy was not associated with increased rates of second leukemias, which could be attributed almost entirely to alkylating agent treatment. A joint monograph on multiple primary cancers, focusing on long-term survivors, was published in collaboration with the Connecticut Tumor Registry and the Danish Cancer Registry. Cancer patients in Connecticut were found to have a 31% increased risk of developing a second primary cancer, which rose to 49% among those surviving more than 30 years. Some constellations of cancers appeared to be due to smoking or alcohol (e.g., lung, larynx, esophagus, buccal cavity, and pharynx), whereas others seemed to be related to hormonal or dietary factors (e.g., colon, uterine corpus, breast, and ovary). In some instances, second cancers appeared to be caused by radiotherapy (e.g., rectal cancer following cancers of the female genital tract, and leukemia following uterine corpus cancer) or by chemotherapy (e.g., acute nonlymphocytic leukemia following multiple myeloma, Hodgkin's disease, and cancers of the breast and ovary). The nonneoplastic effects of radiotherapy were examined in a registry of long-term survivors of childhood cancer at the Dana-Farber Cancer Center. Among Wilms' tumor survivors, an excess of adverse pregnancy outcome was found among the offspring of females receiving abdominal radiation. In a five-center collaborative study designed to evaluate the late effects of childhood cancer treatment, pregnancy rates in survivors were about 30% lower than in controls, with the greatest depression of fertility seen among those receiving radiotherapy combined with chemotherapy.

Studies on Therapeutic Agents

Studies were continued to evaluate the carcinogenic effects of cytotoxic drugs, hormones, and other compounds. A survey of patients given methyl-CCNU, a nitrosourea used in cancer chemotherapy, provided the first quantitative evidence that the risk of developing a leukemic disorder was directly related to the total dose per surface area administered. Another nitrosourea, BCNU, was found to increase the risk of leukemia among patients with brain cancer. Comparative studies of patients treated for ovarian cancer indicate that the leukemogenic potential for melphalan is significantly higher than that for cyclophosphamide. Alkylating agents to treat childhood cancer were associated with an increased risk of leukemia and bone cancer--the latter provides evidence that solid tumors may result from chemotherapy. Thyroid cancer was not increased after treatment with alkylating agents, but synergistic effects were detected when actinomycin D was used with radiation therapy. An analysis of cancer registry data and a pilot study in Connecticut suggested that women with breast cancer who received chemotherapy are prone to leukemia. Data from the Breast Cancer Detection Demonstration Project (BCDDP) revealed no association between

ever use of menopausal estrogens and the risk of breast cancer, but a 50% elevation in risk for users of 20 or more years. Case-control studies revealed an increased risk of invasive cervical cancer associated with oral contraceptives, particularly with long-term use. This risk persisted after adjusting for sexual activity and smoking, which were independent risk factors. The use of phenacetin-containing analgesics was implicated in a case-control study of renal pelvis cancer, which also suggested the possible influence of acetaminophen. These associations are being further evaluated by a multicenter case-control study of cancers arising from the renal pelvis and ureter.

Family Studies

Enhanced by collaborative ties with laboratory investigators, epidemiologic and clinical observations have resulted in the delineation of familial cancer syndromes and several leads to mechanisms of host susceptibility. The discovery of the dysplastic nevus syndrome has provided a marker of susceptibility to melanoma, enabling early detection and treatment of this potentially lethal cancer. In 14 families studied intensively over a 7-year period, 51 new primary melanomas have been detected in 23 patients, and all but two were surgically curable. An analysis of the segregation of melanoma and dysplastic nevi in high-risk families indicated an autosomal dominant pattern of inheritance. The suggestion of linkage between the melanoma/DNS locus and the Rh blood group, located on the short arm of chromosome 1, is being further investigated with restriction fragment length polymorphism analysis to try to map the gene. Lymphocytes from patients with melanoma and dysplastic nevi have shown increased mutability following exposure to UV light. In routine cytogenetic studies, family members with melanoma and dysplastic nevi had an increased frequency of chromosome breaks, which suggests a chromosome instability component to the syndrome.

Studies of a familial syndrome featuring soft-tissue sarcoma, breast cancer, and other neoplasms have led to the discovery of *in vitro* cellular radioresistance, which is being further investigated. Cytogenetic studies of sporadically occurring synovial sarcoma have revealed a specific translocation involving the X chromosome and chromosome 18, an observation with etiologic and diagnostic implications. Complex segregation analysis of 11 Hodgkin's disease families revealed a genetic model that was intermediate between recessive and dominant models. A reanalysis of relative pairs with Hodgkin's disease suggested that approximately 60% of cases are due to a susceptibility allele closely linked to the HLA region. The association with DQ1 has persisted in all the families tested to date. The clinical evaluation of seven families with nevoid basal cell carcinoma syndrome has been completed, and cytogenetic and linkage studies suggest two specific areas of the genome as likely candidates for the gene location. In a family prone to renal adenocarcinomas and a 3:8 constitutional chromosome translocation, the breakpoint on chromosome 8 was the band q24 which contains the *c-myc* oncogene. Molecular studies, however, revealed that the breakpoint was more than 19 kb from the oncogene locus. In a series of nonfamilial renal cancers, the most common cytogenetic changes in the tumors (mainly deletions or translocations) involved the p14-21 region of chromosome 3. This is where the chromosome 3 breakpoint occurred in the constitutional translocation detected in the high-risk family. A polymorphic probe has been localized

to this region and will be used to test the hypothesis that loss of heterozygosity for 3p14-21 is relevant to renal carcinogenesis. The repository of cancer-prone families in the Program has become of increasing interest to experimentalists involved in the identification of human oncogenes, and tissue specimens are made available upon request to the extramural community.

Biostatistics: Continued emphasis was given to the development of basic and applied statistical methodology with applications to several areas, notably epidemiology and carcinogenesis research. New avenues of statistical research were initiated to aid in evaluating the natural history of AIDS and assessment of the reproducibility of laboratory tests for detecting antibodies to the causal agent, HTLV-III/LAV. Improved methods were developed for finding confidence intervals for ratios of proportions used in estimating attributable risk in case-control studies and relative risk in cohort studies. In addition, simplified formulae were derived for estimating sample size requirements for detecting linear trends and for assessing differences in relative risk associated with continuous vs. binary exposure variables. The Program cosponsored a conference on the issue of time-related aspects of human carcinogenesis. Two staff members contributed extensively to the development of congressionally mandated "radioepidemiology tables," to be used as a guide for the probability of radiation being responsible for cancers among persons exposed to radioactive fallout. Two others evaluated, in detail, the statistical issues of carcinogenicity testing for a monograph on this topic to be published by the International Agency for Research on Cancer.

Activities in the Office of the Director

The Division of Cancer Etiology is responsible for planning and directing a national program of basic research including laboratory and epidemiologic studies on the causes and natural history of cancer; basic research on methods and approaches to cancer prevention is also within the Division's sphere of activities. These research efforts are carried out in the intramural laboratories and branches of the Division as well as extramurally, utilizing research grants, cooperative agreements, interagency agreements and contracts. The Office of the Director coordinates, plans and directs a program of national and international research in cancer etiology and also serves as a focal point for the Federal government for the synthesis and dissemination of clinical, epidemiological and experimental data related to cancer etiology and cancer prevention.

Activities in the area of environmental carcinogenesis are located in the Office of the Director. A number of cooperative projects and collaborations with other Federal agencies are carried out under interagency agreements with the U.S. Environmental Protection Agency (EPA), National Institute for Occupational Safety and Health (NIOSH), and National Oceanic and Atmospheric Administration (NOAA). In addition to managing and serving as Project Officers on these interagency agreements, staff from the Office of the Director interface with state agencies, industrial and trade organizations, academic institutions and professional societies, serving a primary role in dissemination of information on environmental problems and industrial exposures in carcinogenesis.

The Interagency Collaborative Group on Environmental Carcinogenesis (ICGEC), organized within the Office of the Director 13 years ago, also serves as a vehicle for information exchange. The ICGEC was originally constituted to provide a mechanism for interagency contacts to secure access to data bases; it has provided, indirectly, a stimulus for development of projects in the area of environmental and occupational carcinogenesis. It consists of representatives from 28 agencies or subagencies, and meets every 2-3 months. By October 1986 there will have been 83 meetings. Topics of meetings held so far this year are as follows: (1) In Vivo and In Vitro Respiratory Tract Carcinogenesis Relevant to Lung Cancer in Man, (2) In Vitro Testing, (3) Current Activities in the Study of Mesothelioma and Lung Cancer.

Staff in the Office of the Director actively participate on the Task Force on Environmental Cancer and Heart and Lung Disease, for which EPA is the lead agency. This Task Force was formed some years ago in response to Congressional stimulation as a result of mandates under the Clean Air Act. A Congressionally mandated DHHS report entitled "Research Activities of Relevance to the Clean Air Act: Biennial Report to Congress" is prepared by the Office of the Director every 2 years.

The Office of the Director supports, by staff and by contractor, the NCI Chemical Selection Working Group (CSWG) for NCI nominations of chemicals to the National Toxicology Program (NTP). It also maintains a Chemical Selection Planning Group (CSPG) which works with a contractor to develop nominations and make decisions on chemicals to be submitted to the CSWG.

Another information dissemination activity involves preparation, under contract, of the "Survey of Compounds Which Have Been Tested for Carcinogenic Activity". Previous contracts provided for the preparation of volumes for 1974-75, 1976-77, and 1979-80, which have been distributed. The present contract, with Technical Resources, Inc., provides for the preparation of volumes for 1981-86. The contractor has completed the 1983-1984 volume; a search of on-line data bases and manual screening resulted in entries on 817 chemicals extracted from 1,097 articles selected from 592 journals published during 1983-84. The volume totals 2,062 pages. The contractor has continued, at no extra cost to the government, with the expanded column "Detailed Information," which now includes authors' comments and conclusions, explanatory notes, and references to other citations. The 1983-84 volume is currently undergoing NIH clearance prior to publication by the Government Printing Office (GPO). Also during this reporting period, the 1981-82 volume was received from GPO and distributed to a large, worldwide user group. The results from a recent user survey indicate that this document continues to be a valuable resource to Federal, state and local health departments and regulatory agencies, as well as investigators involved in research on environmental cancer. These reports, as well as the IARC (International Agency for Research on Cancer) Monographs are distributed through the Office of the Director.

International Agency for Research on Cancer (IARC) Monograph Series "Evaluation of the Carcinogenic Risk of Chemicals to Humans"

The Division supports a Cooperative Agreement with IARC which is managed by staff of the Office of the Director. IARC is located in Lyon, France and the title of the project is "Evaluation of the Carcinogenic Risk of Chemicals to Humans." IARC established this program in 1970 and monographs have been published in volumes so entitled for a large number of chemicals. Thus far 37 volumes have been published and several are in production; the volumes contain monographs in which the carcinogenic risk to man of chemicals, groups of chemicals and, more recently, of industrial and occupational exposures, as well as life-style factors, is evaluated on the basis of results in experimental animals, studies in in vitro systems and epidemiologic studies. The monographs also contain background information on the chemicals under consideration such as chemical and physical properties, analysis, occurrence, production, use and estimated human exposures from all sources. This information is provided to IARC by NCI through a resource contract currently held by Tracor-Jitco. The IARC monographs have become a highly respected and authoritative reference source for countries around the world. Another IARC activity supported under this agreement is the compilation of a listing of laboratories around the world into a compendium entitled "Survey of Chemicals Being Tested for Carcinogenicity." The IARC initiated this survey in 1973 on a worldwide basis; thus far 11 surveys have been published and the twelfth survey is in preparation. These surveys are made available so that laboratories involved in carcinogenesis research can coordinate their testing and research, thus avoiding unnecessary duplication.

Registry of Tumors in Lower Animals

The Division continues to support the Registry of Tumors in Lower Animals (RTLA) which is located at the Smithsonian Institution in Washington, D.C. The RTLA

is the focal point through which information on neoplasms in lower animals is channelled and maintained. Neoplasms and tumor-bearing animals of invertebrate or cold-blooded vertebrate species are collected, studied, classified and preserved at the Registry, which maintains the largest collection of lower animals in the world. In addition to maintaining a specimen depository, the RTLA provides a diagnostic service to biologists in many fields and consequently assists in the identification of clusters of neoplasms in feral animals that may have been exposed to environmental carcinogens in their habitat. Another ongoing activity of the RTLA is the collection and indexing of all scientific literature pertinent to neoplasia in lower animals, including experimentally induced, genetically influenced and "spontaneous" tumors. Together with a computerized listing of the Registry's specimen accessions, this constitutes virtually all the information available on neoplasms in lower animals.

Special Projects on Environmental Carcinogenesis

Centers for Disease Control: Studies on the Human Health Consequences of Polybrominated Biphenyls (PBBs) Contamination of Farms in Michigan

The Center for Environmental Health Sciences, Michigan Department of Public Health, continues to successfully maintain the cohort of exposed Michigan farmers and consumers that were contaminated as a result of the animal feed error of 1973. The emphasis on cohort maintenance continued to be the main thrust, and consequently the "dropout" rate, other than that due to death, continues to be at the nominal rate of 0.5%. Computerization of the large volume of data collected has resulted in significant improvement in efficiency. A newsletter dedicated to this activity is being widely circulated throughout the State of Michigan to enhance visibility and viability of this project. The enrolled cohort is being used in a series of collaborative grants, and other grant proposals are in various stages of development.

Environmental Protection Agency (EPA): Performance of Collaborative Studies in the Area of Environmental Cancer

Due to funding restrictions, no new projects were started under this interagency agreement. A highly successful Fourth NCI/EPA/NIOSH Collaborative Workshop: Progress on Joint Environmental and Occupational Cancer Studies was held April 22-23, 1986, at the Holiday Inn Crowne Plaza in Rockville, Maryland. The capacity audience participated fully in this activity. Of the 130 attendees, over 40 were nongovernment, representing a spectrum of academia, trade associations, and private companies who had interest in one or more of the presentations. Following this workshop, on April 23, 1986, an informal NCI/EPA workshop was held to explore new areas for collaboration and information exchange between NCI/DCE's Epidemiology and Biostatistics Program and EPA's Monitoring Program and Health Research Laboratories.

National Institute for Occupational Safety and Health (NIOSH): Conduct of Research on Occupational Carcinogenesis

A major epidemiological study entitled "Occupational Cancer in Workers Exposed to Silica and Asbestos in the North Carolina Dusty Trade Industries" has been

initiated successfully. Additional funding was placed in the "Ethylene Oxide Mortality Study" initiated by NIOSH as a consequence of the cohort enlargement. NCI and NIOSH staff are collaborating closely on the project "Industry and Occupation Coding of Death Certificates" which is of significant mutual interest. A highly successful Fourth NCI/EPA/NIOSH Collaborative Workshop: Progress on Joint Environmental and Occupational Cancer Studies was held April 22-23, 1986, at the Holiday Inn Crowne Plaza in Rockville, Maryland. The capacity audience participated fully in this activity. Of the 130 attendees, over 40 were non-government, representing a spectrum of academia, trade associations, and private companies who had interest in one or more of the presentations.

National Oceanic and Atmospheric Administration: Etiology of Tumors in Bottom-Dwelling Marine Fish

All field study activities under this interagency agreement have been completed. Correlation has been achieved between mutagenic elements in sediment samples and residues in fish from the affected areas. The laboratory studies are in the final phase. After difficult pioneering efforts to utilize juvenile marine flatfish as a laboratory animal model, a successful bioassay utilizing a diet exposure is well underway, as are egg inoculation studies of sediment extracts in rainbow trout.

Gulf Coast Research Laboratory: Biochemical, Pharmacological and Tumorigenic Studies on a Composite of Drinking Water Carcinogens and Mutagens Utilizing Aquatic Animals as a Bioassay System

The major structural damage and complete disruption of ongoing major bioassays caused by Hurricane Elena in the last quarter of FY 85 have been repaired and reinstated. The contractor has successfully demonstrated the sensitivity of three small fish species to classical carcinogens and to inadvertent drinking water contaminants caused by the chlorination process. The results of this study have been well publicized in presentations and in the open literature, and the methodology developed under this contract is or will be used by a wide variety of organizations. The Department of Defense and EPA are closely monitoring the progress of this activity, and the American Petroleum Institute has awarded a similar contract to this facility with a workscope patterned closely after this contract.

Microbiological Associates, Inc.: In Vitro Evaluation of Chemical Candidates for In Vivo Testing -- Mouse Lymphoma Assay and Salmonella Typhimurium Assay

Some 60 compounds have been tested in the first 2 years of these 3-year contracts, and the results have been utilized by intramural scientists and the chemical selection process for nominations to the National Toxicology Program. The exchange of data with other Federal agencies, i.e., the U.S. Army Medical Bioengineering Research and Development Laboratory and the Food and Drug Administration, continues to be of mutual benefit. Any positive results produced by this activity are incorporated into the Chemical Carcinogenesis Research Information System (CCRIS) data base, which is now available internationally through the National Library of Medicine's TOXNET system. Results of both assays are being incorporated into a series of manuscripts that have

been or will be published in the open literature. The results gained from these two contracts, as well as a previous 3-year contract, are being analyzed for an interassay comparison study. Because of the wide spectrum of chemical structures embodied in 175 compounds, there appears to be an opportunity to examine these assays and their reliability based on each assay's interaction with specific structures.

Tracor Jitco, Inc.: A Resource to Support the Chemical, Economic, and Biological Information Needs of the Division of Cancer Etiology and to Provide Chemical Process, Production and Economic Information to the International Agency for Research on Cancer

During this reporting period, the contractor prepared summary sheets on 33 chemicals which were considered for nomination for carcinogenicity testing, by the National Toxicology program, during four separate meetings of the CSWG. A class study of oximes was performed with special emphasis on environmental and human exposure to these compounds. Monthly meetings of the Chemical Selection Planning Group were held to select the best possible candidates for consideration by the CSWG. The contractor provided support for IARC working group meetings on: Some Naturally Occurring Substances, Food Additives and Amino Acid Pyrolysates in Foods; Halogenated Hydrocarbon and Pesticide Exposure; and Silica and Silicate Compounds. The Chemical Carcinogenesis Research Information System data base was reformatted and updated and is now included in the National Library of Medicine's nationally and internationally available TOXNET system as well as three commercially available data systems.

Chemical Carcinogenesis in Nonhuman Primates

Staff of the Office of the Director direct a large project on chemical carcinogenesis in nonhuman primates. Twenty-eight substances, including antitumor agents, contaminants of human food-stuffs, rodent carcinogens, pesticides, and artificial sweeteners have been or are being evaluated in four species of nonhuman primates for their potential carcinogenicity and other long-term toxic effects. Seventeen of these substances have not as yet demonstrated carcinogenic activity, although some have been on test for less than 4 years. Eleven of the compounds are carcinogenic in nonhuman primates, producing tumors in 5 - 100% of the treated animals. 1-Methyl-1-nitrosourea induced squamous cell carcinomas of the oropharynx and esophagus, with the esophageal tumors possessing clinical and morphologic similarities to human esophageal carcinoma. Long-term treatment with procarbazine resulted in an increased incidence of malignancies, one-half of which were acute nonlymphocytic leukemia. The effects of six of the compounds (DENA, DPNA, 1-nitrosopiperidine, aflatoxin B₁ MAM-acetate and urethane) were manifested primarily as hepatocarcinogenicity. Single cases of malignant tumors have been diagnosed in animals treated with adriamycin (acute myeloblastic leukemia), butter yellow (bronchioalveolar carcinoma), sterigmatocystin (hepatocellular carcinoma) and cyclophosphamide (transitional cell carcinoma of the urinary bladder).

OFFICE OF THE DIRECTOR
DIVISION OF CANCER ETIOLOGY

CONTRACTS ACTIVE DURING FY 86

<u>Institution/Principal Investigator/ Contract Number</u>	<u>Title</u>
Centers for Disease Control Matthew Zack Y01-CP-60215	Studies on the Human Health Consequences of Polybrominated Biphenyls (PBBs) Contamination of Farms in Michigan
Environmental Protection Agency W. Farland and F. Ulvedal Y01-CP-80205	Performance of Collaborative Studies in the Area of Environmental Cancer
National Institute for Occupational Safety and Health Roy M. Fleming Y01-CP-60505	Conduct of Research on Occupational Carcinogenesis
National Oceanic and Atmospheric Administration Donald C. Malins Y01-CP-40507	Etiology of Tumors in Bottom-Dwelling Marine Fish
Gulf Coast Research Laboratory Robin Overstreet N01-CP-26008	Biochemical, Pharmacological and Tumorigenic Studies on a Composite of Drinking Water Carcinogens and Mutagens Utilizing Aquatic Animals as a Bioassay System
Microbiological Associates, Inc. Andrea Back N01-CP-41004	In Vitro Evaluation of Chemical Candidates for In Vivo Testing -- Mouse Lymphoma Assay
Microbiological Associates, Inc. Steve Haworth N01-CP-41030	In Vitro Evaluation of Chemical Candidates for In Vivo Testing -- Salmonella Typhimurium Assay
Technical Resources, Inc. Anthony Lee N01-CP-15761	Survey of Compounds Which Have Been Tested for Carcinogenic Activity
Tracor Jitco, Inc. Stephen S. Olin N01-CP-41003	Resource to Support the Chemical, Economic, and Biological Information Needs of the Division of Cancer Etiology (DCE) and to Provide Chemical Process, Production and Economic Information as Support to the IARC (International Agency for Research on Cancer)

Institution/Principal Investigator/
Contract Number

Title

Smithsonian Institution
John Harshbarger
N01-CP-51031

Operation of a Registry of Tumors in
Lower Animals

Hazleton Laboratories
America, Inc.
N01-CP-03509

Induction, Biological Markers and
Therapy of Tumors in Primates

GRANTS ACTIVE DURING FY 86

International Agency for
Research on Cancer
Harri Vainio
5-U01-33193-05

IARC Monographs on the Evaluation
of the Carcinogenic Risk of Chemicals
to Humans

INTERNATIONAL AGREEMENTS AND INFORMATION EXCHANGE ACTIVITIES

(Fiscal Year 1986)

The Division of Cancer Etiology (DCE) participates in several of the major international agreements for cooperation in cancer research: U.S.-Peoples Republic of China (1980); U.S.-Germany (1979); U.S.-Italy (1979); U.S.-Japan (1974); and U.S.-U.S.S.R. (1972). Collaborative efforts include studies in cancer epidemiology and chemical, physical and biological carcinogenesis, with emphasis on factors related to the etiology and prevention of cancer. Basic and applied research also is conducted in foreign institutions under grants, contracts and cooperative agreements administered through the Division's extramural programs.

U.S.-People's Republic of China. Cancer epidemiology has been given the highest priority under the U.S./China program, but cooperation extends to areas of molecular biology and other disciplines in cancer etiology. Collaborative epidemiologic studies to identify the environmental determinants of esophagus, lung, and stomach cancers and choriocarcinoma drew near completion this year in four areas of China at high risk for these malignancies. In addition, a new collaborative investigation was launched in Shenyang to evaluate air pollution from industrial sources and home heating as a risk factor for lung cancer. The studies are supported in part by contracts from the Division of Cancer Etiology, and were initiated after pilot studies demonstrated their feasibility. A vitamin intervention trial also was initiated in an area of north central China where rates from esophageal cancer are the highest in the world and where there are chronic deficiencies of several micronutrients. Collaborative laboratory research continued during the year. Progress was made on establishing culture conditions for human liver and esophagus, studying the metabolism of chemical carcinogens, assessing biochemical and immunochemical markers in persons at high risk of liver, esophagus, stomach, and lung cancers, and investigating in vitro transformation of human epithelial cells by microbial and chemical agents.

SCIENTIST EXCHANGES

U.S. to China:

Applicant (Laboratory)	Recipient (Laboratory)	Duration	Title of Research
Dr. G. Yoakum Dr. C. Harris National Cancer Inst. (Bethesda, MD)	Drs. T. Sun and C. Hsia Cancer Institute (Beijing)	1.5 months	Investigations of Microbial Agents and Chem- icals in Human Carcinogenesis

China to U.S.:

Dr. C. Hsia Dr. T. Sun Dr. M. Wu Cancer Institute (Beijing)	Dr. C. Harris National Cancer Inst. (Bethesda, Md.)	3 months	Risk Factors for Liver and Esophageal Cancers
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U.S.-Federal Republic of Germany. Substantial progress has been made during the past year in reactivating this program. A bilateral workshop entitled "The Role of DNA Amplification in Tumor Initiation and Promotion" will convene in Heidelberg in October of 1986. It is anticipated that this workshop will form the basis for active scientific collaborations between German and American workers in this rapidly developing area of research. In addition, a number of research areas for collaborative projects have been developed for presentation at the next meeting of the Senate Commission for Cancer Research of the DFG.

U.S.-Italy Agreement. Research pertinent to this Division is included in the Cancer Prevention area of the U.S.-Italy Cancer Program. The program involves a variety of activities related to cancer etiology, with recent emphasis on epidemiology. This year a collaborative case-control study of gastric cancer, the leading cause of cancer death in parts of northern and central Italy, was begun. Stimulated by workshops organized under the Agreement, the multi-center stomach cancer investigation involves high and low risk areas of Italy and will be funded in part by a contract from the Division of Cancer Etiology with the Center for Study and Prevention of Cancer in Florence. During the past year the Division has been developing a new initiative in the area of perinatal carcinogenesis. A joint U.S.-Italy workshop entitled "Transplacental Etiology of Human Neoplasms" is in the planning stage and will be convened in Genoa in November of 1986. The purpose of the workshop is to bring together epidemiologists and experimentalists who are interested in the etiology of pediatric neoplasms and other forms of human cancer, and who have an interest in human transplacental exposures to environmental carcinogenic agents. It is anticipated that this workshop will serve as a stimulus for future collaborative research between American and Italian scientists.

U.S.-Japan Agreement. This year marks the second year of the third 5-year program of this agreement, which consists of four broad program areas: Etiology, Cancer Biology and Diagnosis, Cancer Treatment, and Interdisciplinary Research. The cooperation between U.S. and Japanese scientists still remains one of the most active and is especially well suited to the study of malignancies that differ markedly in their occurrences within the two nations. Within the Etiology Area of the U.S.-Japan Agreement, seminars were held on "Oncogenes and Experimental Carcinogenesis," "Causative and Modifying Factors in Digestive Tract Cancer," and "Oxygen Radicals in Cancer."

Five scientists from Japan participated in exchange programs in Etiology Research which has as its mission to provide a fundamental basis for understanding cancer causation that, in turn, would identify effective means for preventing or modulating this process.

SCIENTIST EXCHANGES

Japan to U.S.:

Applicant (Laboratory)	Recipient (Laboratory)	Duration	Title of Research
Dr. Shun Nakamura The University of Tokyo (Tokyo)	Dr. G. Vande Woude Litton-Bionetics, Inc. (Frederick, Maryland)	3 months	Development of Retrovirus Vector Systems and its Application for the Study of the Function of Onco- genes
	Dr. E. Gilboa Princeton University (Princeton, New Jersey)		
	Dr. K. Arai DNAX Research Institute		
Dr. Masuo Yutsudo National Cancer Institute (Suita)	Dr. Peter M. Howley National Cancer Inst. (Bethesda, Md.)	1 month	Mechanism of Tumorigenicity by Human Papilloma- virus
Dr. Tada-Aki Hori Natl. Institute of Radiological Sciences (Chiba)	Dr. Thomas W. Glover University of Michigan (Ann Arbor, Michigan)	1 month	Studies on Mechanisms of the Expression of Heritable Fragile Sites on Human Chromosomes
Dr. Yuji Kurokawa Natl. Institute of Hygienic Sciences (Tokyo)	Dr. Thomas J. Slaga The University of System Cancer Center (Houston, Texas)	2 weeks	Carcinogenicity Studies of Environ- mental Chemicals in vivo and in vitro
Dr. Kazuo Negishi Okayama University (Okayama)	Dr. Robert D. Wells University of Alabama at Birmingham (Birmingham, Alabama)	2 months	Carcinogenesis and Z-form DNA

U.S.-U.S.S.R. Agreement. We have continued our attempt to redefine and restructure the American-Soviet Cooperative Program in Carcinogenesis. Since this represents a new field of scientific endeavor in the U.S.-USSR Cooperative Cancer Program, we deem it essential to obtain from the USSR as much information as possible on the ongoing work in research areas that were identified as being potentially beneficial to both nations. These include: (1) modifying effects of chemicals on gene expression of normal and neoplastic cells; (2) role of tumor promoters in biological, chemical, and physical carcinogenesis; (3) genetic analysis of malignancy by means of somatic cell hybridization; (4) chemical induction of tumors in specific target organs; (5) gene regulation and gene amplification relating to viral and chemical carcinogenesis; (6) the role of viral-related transforming (onc, sarc) genes in the genesis of spontaneous and induced tumors of animals and man; (7) development and exchange of monoclonal antibodies (hybridomas) directed against different antigens in biologically- and chemically-induced tumors; (8) studies on the development of cancers resulting from transplacental and perinatal exposures to biological and chemical carcinogens; (9) chemical/viral cocarcinogenesis studies in primates; (10) genetic disorders with predisposition to malignancy; (11) mutagenic action of anticancer drugs; (12) clinical aspects of somatic cell genetics; and (13) studies on new candidate oncogenic virus isolates from primates, including man. We have asked for the following information: (1) the number of research groups or institutions and their location in the Soviet Union where scientific effort is underway in the areas we listed; (2) the principal scientists that participate in these activities, their affiliation and a bibliographic listing of their scientific contributions in areas of carcinogenesis; and (3) the availability of Soviet principal scientists in carcinogenesis for in-depth discussion of research interests and results for consideration of carcinogenesis problem areas amenable to joint investigation. We are awaiting this information in order to plan collaboration and/or selection of American scientists unique and appropriate to the research areas in which American and Soviet specialists will interact.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01CP03509-23 OD

PERIOD COVERED

October 1, 1985 to September 30, 1986

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Carcinogenesis, Chemotherapy and Biological Markers in Nonhuman Primates

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: S. M. Sieber Deputy Director OD, DCE NCI
 Others: J. Whang-Peng Head, Cyto. Oncology Section MB NCI

COOPERATING UNITS (if any)

Department of Pathology, Louisiana State University, New Orleans, LA (P. Correa);
 Hazleton Laboratories America, Inc., Vienna, VA (D. Dalgard, R. J. Parker)

LAB/BRANCH

Division of Cancer Etiology

SECTION

Office of the Director

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

4.0

PROFESSIONAL:

1.5

OTHER:

2.5

CHECK APPROPRIATE BOX(ES)

- (a) Human subjects (b) Human tissues (c) Neither
 (a1) Minors
 (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

A wide variety of substances, including antitumor and antineoplastic agents; food additives, food components and environmental contaminants; "model" rodent carcinogens; and nitroso- compounds have been or are being evaluated in four species of nonhuman primates for their potential carcinogenicity and other long-term toxic effects. Of the 29 test compounds, 16 have not as yet demonstrated carcinogenic activity, although some have been on test for less than 4 years. Nine of the compounds are carcinogenic in nonhuman primates, producing tumors in 10-100% of the treated animals. 1-Methyl-1-nitrosourea (MNU) induced squamous cell carcinomas of the oropharynx and esophagus, with the esophageal tumors possessing clinical and morphologic similarities to human esophageal carcinoma. Long-term treatment with procarbazine resulted in an increased incidence of malignancies, one-half of which were acute nonlymphocytic leukemia. The effects of seven of the compounds (DNA, DPNA, 1-nitrosopiperidine, aflatoxin B-1, MAM-acetate, urethane and sterigmatocystin) were manifested primarily as hepatocarcinogenicity. Single cases of malignant tumors have been diagnosed in animals treated with adriamycin (acute myeloblastic leukemia), butter yellow (bronchioalveolar carcinoma), cyclophosphamide (transitional cell carcinoma of the urinary bladder), and 3-methyl-DAB (hepatocellular carcinoma).

PROJECT DESCRIPTIONNames, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

S. M. Sieber	Deputy Director	OD, DCE	NCI
J. Whang-Peng	Head, Cyto. Oncology Section	MB	NCI

Objectives:

One of the objectives of this project is to obtain comparative data on the response of nonhuman primates to known rodent carcinogens as well as to materials suspected of being human carcinogens. Of particular interest in this regard is to evaluate, in monkeys, several chemicals of social or economic importance (e.g., DDT, saccharin, cyclamate, arsenic) and for which rodent data are difficult to interpret or are contradictory. Another objective of the project is to evaluate the long-term toxic effects (including carcinogenicity) of antineoplastic agents which currently are in clinical use for primary and adjuvant cancer therapy and for treatment of an increasingly broad spectrum of nonmalignant diseases. Other objectives include obtaining model tumor systems in primates for use in testing the effectiveness of new antitumor agents, developing models and strategies for chemoprevention, and developing biological markers and diagnostic tests for early detection of tumors. In addition, this project makes available normal and tumor-bearing animals for a variety of pharmacologic, toxicologic, biochemical and immunological studies. The maintenance of a breeding colony ensures that offspring will be available for use.

Methods Employed:

Twenty-nine substances are currently under investigation or have been investigated, including antineoplastic and immunosuppressive agents (procarbazine, adriamycin, MNU, melphalan, azathioprine and cyclophosphamide), food additives, food components and environmental contaminants (aflatoxin B₁, methylazoxymethanol-acetate, sterigmatocystin, IQ, cyclamate, saccharin, butter yellow, 3-methyl-DAB, DDT, arsenic and cigarette smoke condensate), "model" rodent carcinogens (urethane, 3-MC, 2-AAF, 2,7-AAF, copper chelate of N-OH-AAF, dibenzpyrene and dibenzanthracene), and various nitroso- compounds (DMNA, DENA, DPNA, 1-nitrosopiperidine and MNNG).

The compounds are administered subcutaneously, intravenously, intraperitoneally, or orally. For oral administration to newborn monkeys, the compound was added to the Similac formula at the time of feeding; when the monkeys are 6 months old, carcinogens given orally are incorporated into a vitamin mixture which is given to monkeys as a vitamin sandwich on a half slice of bread. An alternate way of giving doses orally is to incorporate the compound into baited foods or to administer it by intubation. The dose level chosen is dependent on the

chemical under evaluation. Antineoplastic and immunosuppressive agents are administered at doses likely to be encountered in a clinical situation; other substances, such as environmental contaminants, are given at levels 10 to 40-fold higher than the estimated human exposure level. The remainder of the chemicals tested are administered at maximally tolerated doses which, on the basis of weight gain, blood chemistry and hematology findings, and clinical observations, appear to be devoid of acute toxicity.

The present colony, consisting of 494 animals, is comprised of four species: Macaca mulatta (rhesus), Macaca fascicularis (cynomolgus), Cercopithecus aethiops (African green) and Galago crassicaudatus (bushbabies). Sixty-six of these monkeys are adult breeders which supply juvenile animals for experimental studies. The majority of the animals are housed in an isolated facility which contains only animals committed to this study, and with the exception of the breeding colony, most animals are housed in individual cages. The administration of test compounds is continued until a tumor is diagnosed or until a predetermined exposure period is completed. A minimum of 30 animals is usually allotted to each treatment group, since in a sample of this size it is possible to detect a tumor incidence of 10% within 95% confidence limits.

A variety of clinical, biochemical and hematological parameters are monitored weekly or monthly, not only to evaluate the general health status of each animal, but also for the early detection of tumors. Surgical procedures are performed under Ketamine or sodium pentobarbital anesthesia. All animals which die or are sacrificed are carefully necropsied and the tissues subjected to histopathologic examination.

Major Findings:

The six chemicals on test, categorized as antineoplastic and immunosuppressive agents, are procarbazine, MNU, adriamycin, melphalan, azathioprine and cyclophosphamide. Both procarbazine and MNU are carcinogenic in monkeys, inducing tumors in approximately one-third of the animals autopsied thus far. One-half of the tumor-bearing animals in the procarbazine study developed acute nonlymphocytic leukemia; the other half developed solid tumors at a variety of sites. MNU was administered orally and induced squamous cell carcinoma of the mouth, pharynx and/or esophagus. Two of the chemicals in this series have induced neoplasms in only one animal thus far. In a group of 10 animals receiving monthly IV doses of adriamycin (1.0 mg/kg), 9 developed congestive heart failure and died or were sacrificed in moribund condition; the tenth animal was diagnosed with acute myeloblastic leukemia. This study is being repeated using lower doses of adriamycin to evaluate further its leukemogenic potential. One of a group of 23 animals receiving cyclophosphamide developed transitional cell carcinoma of the urinary bladder. Animals in this study have been receiving cyclophosphamide for approximately 6 years. The other two chemicals in this series, melphalan and azathioprine, have been on test for approximately 6 and 10 years, respectively, but have provided no evidence of carcinogenicity.

The chemicals categorized as food additives, food components and environmental contaminants are the fungal products, aflatoxin B₁ and sterigmatocystin; MAM-acetate, the synthetic aglycone of cycasin; the artificial sweeteners, cyclamate and saccharin; the azo dyes, butter yellow and its 3-methyl derivative; and the pesticides, DDT and arsenic. Both of the fungal products are carcinogenic in monkeys, inducing hepatocellular carcinoma in 59% (aflatoxin B₁) and 13% (sterigmatocystin) of the animals autopsied thus far. MAM-acetate is also a hepatocarcinogen, inducing tumors in 37% of autopsied monkeys. One animal each in both groups receiving the azo dyes developed a tumor. The tumors, a bronchio-alveolar carcinoma in a monkey treated with butter yellow and a hepatocellular carcinoma in an animal treated with 3-methyl-DAB, developed in relatively old monkeys and more than 15 years after the last exposure to the test compounds. Thus these tumors may be spontaneous rather than chemically-induced. The remainder of the test chemicals in this series have not provided any evidence of carcinogenicity, despite the fact that most have been on test for relatively prolonged periods. For example, the saccharin and cyclamate studies have been in progress for 15 years, the DDT and arsenic studies for 9 and 16 years, respectively. The most recently initiated study in this series, an evaluation of the carcinogenicity of IQ (2-amino-3-methyl-3H-imidazo[4,5-f]quinoline), a heterocyclic amine isolated from pyrolysates of amino acids and proteins, has been underway for less than a year.

The chemicals categorized as "model" rodent carcinogens are urethane, 3-methyl-cholanthrene, 2-acetylaminofluorene (2-AAF), 2,7-AAF, the copper chelate of N-hydroxy-AAF and benzpyrene. Of these chemicals only urethane has proved to have carcinogenic activity in monkeys. Tumors have been detected in 20% of the animals necropsied thus far; many of the animals had multiple primary tumors which in most cases included liver hemangiosarcomas. The tumors were diagnosed after an unusually lengthy latent period which averaged 14 years. The surviving animals in all the test groups in this series have been under observation for tumor development for at least 20 years.

The nitroso- class of chemicals tested include dimethyl-, diethyl-, and dipropyl-nitrosamine, N-nitrosopiperidine and N-methyl-N'-nitro-N-nitrosoguanidine (MNNG). As a general class they are active hepatocarcinogens in nonhuman primates. Dimethylnitrosamine did not induce liver tumors; the high level of liver toxicity exerted by this compound and the associated early mortality probably precluded liver tumor development. The other two nitrosamines, DENA and DPNA, were potent hepatocarcinogens capable of inducing primary hepatocellular carcinoma in virtually every animal exposed. MNNG has not as yet provided evidence of having carcinogenic activity in nonhuman primates; it has been under evaluation for 12-14 years, although only recently has the dose been increased to the range shown to induce tumors in other species such as dogs and rodents.

Publications:

None

Patents:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
 NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP04548-14 OD

PERIOD COVERED

October 1, 1985 to September 30, 1986

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Registry of Experimental Cancers/WHO Collab. Ctr. for Tumours of Lab Animals

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)

P.I.:	Harold L. Stewart	Scientist Emeritus	DCE	NCI
Others:	Bernard Sass	Veterinary Medical Officer	DCE	NCI
	Margaret K. Deringer	Guest Researcher	DCE	NCI
	Carel F. Hollander	Guest Researcher	DCE	NCI
	Annabel G. Liebelt	Guest Researcher	DCE	NCI

COOPERATING UNITS (if any)

LAB/BRANCH

Office of the Director

SECTION

INSTITUTE AND LOCATION

NIH, NCI, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

4.5

PROFESSIONAL:

2.5

OTHER:

2.0

CHECK APPROPRIATE BOX(ES)

- (a) Human subjects (b) Human tissues (c) Neither
 (a1) Minors
 (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The objectives of the Registry of Experimental Cancers are the storage and retrieval of pathologic material and data on cancers and other lesions of laboratory animals (primarily rodents) and the use of such information for research and educational purposes. The Registry has acquired a total of 3,345 (476 since the 1985 report) single or group accessions from investigators outside the NCI and approximately 63,076 records have been coded. Forty-two investigators have come to the Registry for study and consultation on single or multiple visits.

PROJECT DESCRIPTIONNames, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Harold L. Stewart	Scientist Emeritus	DCE	NCI
Bernard Sass	Veterinary Medical Officer	DCE	NCI
Margaret K. Deringer	Guest Researcher	DCE	NCI
Carel F. Hollander	Guest Researcher	DCE	NCI
Annabel G. Liebelt	Guest Researcher	DCE	NCI

Objectives:

1) The storage and retrieval of pathologic material and data on cancers and other lesions of laboratory animals (primarily rodents); 2) the use of such pathologic material and data for research and educational purposes.

Methods Employed:

The methods employed in the work of the Registry involve the selection of protocols, pathologic material, including histologic slides, paraffin blocks, and illustrations in the form of lantern slides, gross photographs, and photomicrographs in black and white and in color. The work of the Registry also includes the collection of records of experiments, reprints and references on this material. The Registry of Experimental Cancers possesses a large collection of spontaneous and induced cancers and other lesions. The pertinent information on the collection is indexed and many of the data have been entered into the computer. The Registry accesses material from investigators at NCI, other institutes of NIH, other governmental agencies, industrial laboratories, and universities here and abroad. A total of 3,345 (476 since the 1985 report) single or group accessions from investigators outside of NIH have been processed. The Registry prepares Study Sets of slides, with explanatory notes, relating to particular cancers of rodents.

The Registry has Study Sets of slides on "Comparative Pathology of Hematopoietic and Lymphoreticular Neoplasms," "Induced and Spontaneous Tumors of Small Intestine and Peritoneum in Mice," "Induced Tumors of the Liver in Rats," "Tumors and Non-neoplastic Lesions of the Lungs of Mice," "Mammary Tumors in Mice," "Pulmonary Metastases in Mice," "Neoplastic and Nonneoplastic Lesions of the Lymphoreticular Tissue in Mice," "Neoplasms and Other Lesions of Praomys (Mastomys) Natalensis," "Malignant Schwannomas of Rats," "Harderian Gland Tumors of Mice," "Renal Tumors of Rats," "Spontaneous Gastric Adenomatosis, Polyps and Diverticula; Duodenal Plaques of Mice," "Adrenal Tumors of Mice," and "Schwannomas of Mice." These Study Sets, with descriptive material, are loaned to investigators who request them. Ten loans have been made this year, five of which were to countries abroad.

Investigators come to the Registry for study and consultation. There have been single or multiple consultations with 42 individuals since the 1985 report. Five investigators have sent material to the Registry for diagnosis.

During the period from January 1980 until April 30, 1985, the Registry has received 8,644 requests for reprints of Histologic Typing of Liver Tumors of the Rat (J. Nat'l. Cancer Inst. 64: 177-206, 1980). This histologic classification and typing of rat liver tumors is calculated to promote uniformity of diagnoses from one laboratory to another in this country.

The Director General of the World Health Organization designated the Registry of Experimental Cancers as the WHO Collaborating Centre for Reference on Tumours of Laboratory Animals on October 26, 1976 and PAHO renewed this collaboration on July 19, 1983. This is the only such registry in the world to be so designated by the WHO. The Registry will expand communications between U.S. scientists and those of other countries, now numbering 153, which are members of WHO.

The Registry will expand all of its activities (already set forth in this report).

Major Findings:

The functions (outlined in objectives) of the Registry in the field of cancer research are such that there will be no major findings to report.

Publications:

Hayes, H. M. Jr. and Sass B.: Testicular tumors: Species and strain variations. In Kaiser, H. E. (Ed.): Progressive Stages of Malignant Neoplastic Growth. Boston-Dordrecht-Lancaster England, Martinus Nijhoff. (In Press)

Hoch-Ligeti, C., Liebelt, A. G., Congdon, C. C. and Stewart, H. L.: Mammary gland tumors in irradiated and untreated guinea pigs. Toxicol. Pathol. (In Press)

Hoch-Ligeti, C., Restrepo, C. and Stewart, H. L.: Comparative pathology of cardiac neoplasms in humans and in laboratory rodents: a review. J. Nat'l. Cancer Inst. 76: 127-142, 1986.

Hoch-Ligeti, C. and Stewart, H. L.: Cardiac tumors in laboratory rodent-comparative pathology. In Kaiser, H. E. (Ed.): Progressive Stages of Malignant Neoplastic Growth. Boston-Dordrecht-Lancaster England, Martinus Nijhoff. (In Press)

Liebelt, A. G.: Malignant Neoplasms in Organ Transplant Recipients. In Kaiser, H. E. (Ed.): Progressive Stages of Malignant Neoplastic Growth. Boston-Dordrecht-Lancaster England, Martinus Nijhoff. (In Press)

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Patents:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP06134-11 OD

PERIOD COVERED

October 1, 1986 to September 30, 1986

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Role of Lymphatic System in the Absorption and Distribution of Antitumor Agents

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	S. M. Sieber	Deputy Director	OD, DCE	NCI
Others:	J. N. Weinstein	Senior Investigator	LMB	NCI

COOPERATING UNITS (if any)

Hazleton Laboratories America, Inc., Vienna, VA (R. J. Parker)

LAB/BRANCH

Division of Cancer Etiology

SECTION

Office of the Director

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

2.5

PROFESSIONAL:

1.5

OTHER:

1.0

CHECK APPROPRIATE BOX(ES)

- (a) Human subjects (b) Human tissues (c) Neither
- (a1) Minors
- (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The role of the lymphatic system in the absorption and biodistribution of antitumor agents and monoclonal antibodies in normal and tumor-bearing mice is under investigation. Compared with intravenous administration, monoclonal antibodies given by subcutaneous injection are delivered with higher efficiency to regional lymph nodes where they bind specifically to lymphoid cells. Theoretical studies and large-scale pharmacokinetic studies in mice of both specific and nonspecific monoclonal antibodies, whole antibody and fragments, and several metabolites of iodinated antibodies have now been completed for both intravenous and subcutaneous routes of administration. The pharmacological principles that have emerged from studies in mice are being applied to the design of clinical protocols for the detection of melanoma, T-cell lymphoma, Hodgkin's disease, lung carcinoma and breast carcinoma. Conjugates of anti-B-cell antibody, coupled with an alpha-emitting radiometal bismuth-212, demonstrated specific cytotoxicity in vitro. However, these conjugates were not active in vivo against lymph node metastases of the guinea pig line 10 hepatoma which we attribute to poor immunoreactivity and insufficient radiation dose.

PROJECT DESCRIPTIONNames, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

S. M. Sieber	Deputy Director	OD, DCE	NCI
J. N. Weinstein	Senior Investigator	LMB	NCI

Objectives:

The major objective of this project is to delineate the role of the lymphatic system in the absorption and distribution of antitumor agents and other materials such as monoclonal antibodies following administration by intravenous and subcutaneous routes. Studies are aimed at developing systems for selective delivery of therapeutic or diagnostic agents to lymph nodes harboring metastatic deposits. Investigations are currently focused on optimizing lymphatic delivery of monoclonal antibodies to lymph nodes from which principles may be applied to clinical settings for immunodiagnosis and immunotherapy. Monoclonal antibody conjugates, α -emitting radioisotopes and other cytotoxic agents will be synthesized and their cytotoxicity tested in vitro. Therapeutic protocols will then be developed using the guinea pig L10 tumor system and other animal models of the metastatic process to assess the therapeutic efficacy of such conjugates.

Major Findings:

Data from large-scale biodistribution and metabolic studies in mice, along with data from in vitro antibody binding studies, have been incorporated into physiological models which describe the pharmacokinetics of radiolabelled nonspecific antibody (MOPC), specific anti-B-cell antibody (CY34) and their fragments in vivo. These models suggest the following: 1) The biodistribution of the specific antibody CY34 can be described by the trafficking of the B-cells to which it binds. Uptake of antibody in lymphoid organs such as the spleen may occur primarily by the recruitment of circulating B-cells to which antibody has already bound rather than the uptake of free antibody by B-cells present in these organs; 2) The biodistribution of nonspecific antibody MOPC can be described solely by its distribution in blood, since there is no specific organ uptake for this antibody; 3) Binding of labelled CY34 is associated with an increase in catabolism of the antibody, the major products of which are iodide, arising from dehalogenation of the iodinated protein, and nonacid-precipitable molecules with molecular weights of approximately 1200 daltons; 4) The gastrointestinal tract appears to play a greater role in the catabolism of iodine-labelled antibodies than was previously suspected. Clinical studies utilizing radiolabelled antitumor antibody injected subcutaneously for detection of melanoma were disappointing since little of the antibody was found to localize in regional lymph nodes. In contrast, similar studies for detection of T-cell lymphoma have produced dramatic results with 25-40% of the injected antibody localizing in regional lymph nodes following subcutaneous injection of the Indium-111 labelled

antibody. Conjugates of an antitumor antibody and the α -emitting radio-nuclide bismuth-212 were not effective in treating lymph node metastases of the L10 hepatoma in guinea pigs which we attribute to poor immunoreactivity and insufficient radiation dose. The technique by which conjugates are prepared has now been modified to improve both immunoreactivity and specific activity. Attempts to treat lymph node metastases in guinea pigs will be repeated using these improved conjugates.

Publications:

Covell, D. G., Parker, R. J., Black, C. D. V., Holton, O. D. and Weinstein, J. N.: A kinetic model of monoclonal IgG in mice. Cancer Res. (In Press)

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Patents:

None

ANNUAL REPORT OF
THE LABORATORY OF CELLULAR AND MOLECULAR BIOLOGY
BIOLOGICAL CARCINOGENESIS PROGRAM
DIVISION OF CANCER ETIOLOGY
NATIONAL CANCER INSTITUTE

October 1, 1985 to September 30, 1986

The major goals of the Laboratory of Cellular and Molecular Biology (LCMB) are to determine the etiology of naturally occurring cancer and to elucidate mechanisms of transformation by carcinogenic agents. The ultimate aim of these investigations is to apply approaches successful in animal model systems to the identification of causative agents of human malignancies and to the prevention of cancer in man.

A primary emphasis of many ongoing investigations within the laboratory concerns RNA tumor viruses. These viruses are unique among animal viruses in their mode of transmission and the intimate association that has evolved between these agents and cells of a large number and wide variety of vertebrate species. The etiologic role of this virus group has been established for naturally occurring cancers of many species, including some subhuman primates. Certain members of this virus group, so-called "replication-defective" transforming viruses, have arisen by a recombination with cellular genes or proto-oncogenes. As such, these viruses offer an unparalleled opportunity to elucidate the processes by which such genes cause malignancies. Thus, research within the LCMB encompasses efforts to understand the processes involved in malignancy utilizing RNA tumor viruses as models.

Recent investigations within the laboratory have provided strong evidence that proto-oncogenes are also frequent targets of genetic alterations leading human cells along the pathways to malignancy. Today, much of our research is aimed at identifying genes that can be implicated as oncogenes in human cancer, their molecular mechanisms of activation, as well as the mode of action of their translational products.

During the past year, our laboratory has made progress in several areas, as specified below.

Ras oncogenes and proto-oncogenes. The availability of molecular clones of the normal and activated alleles of human ras proto-oncogenes made it possible to determine the molecular mechanisms responsible for the malignant conversion of these genes. The genetic lesions responsible for activation of a number of ras oncogenes have been localized to single base changes in their p21 coding sequences. Findings during the past year include the following:

Members of the ras gene family are activated as oncogenes in many different human cancers. To systematically determine the frequency at which such genes might be involved in the neoplastic process affecting a specific target tissue, urothelial cells, a large series of urinary tract tumors were surveyed for ras oncogenes by DNA transfection and by molecular genetic analysis. Harvey (H)-ras oncogenes were detected in 2 of 38 tumors by transfection, molecularly cloned in biologically active form, and shown to contain single base changes at

codon 61 leading to substitutions of arginine and leucine, respectively, for glutamine at this position. One additional H-ras oncogene was identified in a bladder carcinoma by restriction polymorphisms at codon 12. In 1 of 21 tumors, a 40-fold amplification of the Kirsten (Ki)-ras gene was detected. No amplification of other ras genes was detected in any of the tumors analyzed. These findings strengthen the conclusion that codons 12 and 61 are the major "hot spots" of ras oncogene activation and suggest that quantitative alterations in expression due to gene amplification may provide an alternative mechanism for ras gene activation in primary human tumors.

Three human ras family proto-oncogenes, c-Ki-ras-1, c-Ki-ras-2, and N-ras, were mapped to chromosome bands 6p11-12, 12p11.1-12.1, and 1p11-13, respectively, by in situ molecular hybridization. Certain human cancers displayed consistent and specific alterations involving chromosomes 1, 16 and 12. The precise chromosomal localization of ras genes permits evaluation of the possible effect of these chromosome changes on the structure and activities of ras proto-oncogenes in human neoplasia.

Using the NIH/3T3 cell transfection assay, activated cellular oncogenes have been detected in around 10% to 20% of human tumors. From a series of DNA preparations from tissues infiltrated with acute myelogenous leukemia (AML), 50% caused transformation of NIH/3T3 cells. Thus, AML appears to be the human tumor with the highest frequency of oncogenes detected by DNA transfection. In each case, the oncogene involved was N-ras, a member of the ras gene family.

Species- and strain-specific spontaneously occurring tumors have been observed in rodents maintained under normal laboratory conditions. Elucidation of the molecular mechanisms associated with the development of these spontaneous tumors may provide a better understanding of tumor development associated with exposure to chemical carcinogens. In view of the high frequency of oncogene activation shown in rodent tumors induced by known chemical carcinogens, oncogene activation in spontaneous tumors of the B6C3F1 mouse and Fischer 344/N rat was investigated by DNA transfection techniques. A marked difference in the presence of activated oncogenes in spontaneous rat tumors versus spontaneous mouse liver tumors was observed. All rat tumors tested failed to yield activated oncogenes, whereas 30% of mouse hepatocellular adenomas and 77% of hepatocellular carcinomas scored positive by DNA transfection. These transforming genes were identified as activated H-ras genes in all the adenoma transfectants. The two remaining hepatocellular carcinomas contained transforming genes that appear not to be members of the known ras gene family. The B6C3F1 mouse liver system might provide a very sensitive assay not only for assessing the potential of a chemical to activate a cellular proto-oncogene, but also for detecting various classes of proto-oncogenes that are susceptible to mutational activation.

Primary cultures of human epidermal keratinocytes grown in NCTC 168 medium supplemented with horse serum, infected with adeno 12-SV40 hybrid DNA viruses, grew, underwent productive and nonproductive transformation and became established lines. The transformed cells contained SV40 large and small tumor antigens but did not contain adenovirus early region (E1A and E1B) messages. The altered cells were not tumorigenic. Subsequent infection of the "flat" nonproducer cells with Ki-MSV produced striking morphological alterations and induced carcinomas when transplanted into nude mice. Thus, neoplastic transformation of primary human epidermal keratinocytes was demonstrated by the

combined action of DNA and RNA tumor viruses. Results establish a multistep process in the progression of human epithelial cells towards malignancy.

The high prevalence of *ras* oncogenes in human tumors has given increasing impetus to efforts aimed at elucidating the structure and function of the p21 products. To identify functionally important domains of the p21 protein, antibodies were generated against synthetic peptides corresponding to various regions of the protein. Antibodies directed against a synthetic peptide fragment corresponding to amino acid residues 161 to 176 in the carboxy-terminal region of the H-*ras*-encoded p21 molecule specifically recognized H-*ras*-encoded p21 proteins. This antibody was also shown to strikingly and specifically inhibit the guanine nucleotide-binding function of the p21 protein. The inability of p21 protein to bind guanine nucleotides was associated with a lack of autophosphorylation or GTPase activities. These studies suggest that a region toward its carboxy terminus is directly or indirectly involved in the guanine nucleotide-binding function of the p21 molecule.

The epitope recognized by Y13-259, a monoclonal antibody which recognizes a shared determinant of *ras* p21 proteins, was localized to a small amino acid stretch that was shown not to be directly responsible for the known biochemical functions of the molecule. Instead, this epitope reflects a new domain critical to the role of p21, the *ras* proto-oncogene product, in the normal pathway leading to DNA synthesis in serum-stimulated cells.

Deletion mutants of the viral Harvey-*ras* oncogene were generated by removing different lengths of the gene from either the amino or carboxy terminus. The deletion mutants, *ras* p21 expressed in *E. coli*, yielded proteins of approximately 8 kd, 10 kd, 12 kd, 14 kd, 17 kd, 18 kd, 19 kd, and 20 kd. These proteins were utilized to identify epitopes recognized by a series of newly generated monoclonal antibodies as well as some previously reported by Furth and associates in 1982. Monoclonals which inhibited GTP binding, a major biochemical activity of the p21 protein, recognized two major domains. These regions were localized from amino acid 5-69 and 107-164, respectively, and were separated by another stretch from residues 70-106, whose antigenic determinants were not directly involved in GTP binding. Thus, the mapping of epitopes within the p21 molecule recognized by monoclonal antibodies has made it possible to localize important functional domains within the *ras* p21 molecule.

Deletion of small sequences from the viral H-*ras* gene have been generated, and resulting *ras* p21 mutants have been expressed in *E. coli*. Purification of each deleted protein allowed the in vitro characterization of GTP-binding, GTPase, and autokinase activities of the proteins. Microinjection of the highly purified proteins into quiescent NIH/3T3 cells, as well as transfection experiments utilizing an LTR-containing vector, were utilized to analyze the biological activity of the deleted proteins. Two small regions, located at 6-23 and 152-165 residues, were shown to be absolutely required for in vitro and in vivo activities of the *ras* product. By contrast, the variable region comprising amino acids 165-184 were shown not to be necessary for either in vitro or in vivo activities. Thus, we demonstrated that (1) amino acid sequences at positions 5-23 and 152-165 of p21-*ras* protein are probably directly involved in the GTP-binding activity, (2) GTP binding is required for the transforming activity of *ras* proteins and, by extension, for the normal function of the proto-oncogenic product, and (3) the variable region at the C-terminus end of the *ras* p21 molecule from amino acids 165 to 184 is not required for transformation.

We sought to determine whether decreased in vitro GTPase activity is uniformly associated with ras p21 mutants possessing efficient transforming properties. Normal H-ras p21-[Gly¹²-Ala⁵⁹] as well as an H-ras p21-[Gly¹²-Thr⁵⁹] mutant exhibited in vitro GTPase activities at least fivefold higher than either H-ras p21-[Lys¹²-Ala⁵⁹] or H-ras p21-[Arg¹²-Thr⁵⁹] mutants. Microinjection of as much as 6×10^6 molecules/cell of bacterially expressed normal H-ras p21 induced no detectable alterations of NIH/3T3 cells. In contrast, inoculation of $4-5 \times 10^5$ molecules/cell of each p21 mutant induced morphologic alterations and stimulated DNA synthesis. Moreover, the transforming activity of each mutant expressed in a eukaryotic vector was similar and at least 100-fold greater than that of the normal H-ras gene. These findings establish that activation of efficient transforming properties by ras p21 proteins can occur by mechanisms not involving reduced in vitro GTPase activity.

v-sis oncogene/platelet-derived growth factor (PDGF)-2. The v-sis transforming gene encodes the woolly monkey homolog of human PDGF-2. After its synthesis on membrane-bound polyribosomes, the glycosylated precursor dimerizes in the endoplasmic reticulum and travels through the Golgi apparatus. At the cell periphery, the precursor is processed to yield a dimer structurally analogous to biologically active PDGF. Small amounts of two incompletely processed forms are detectable in tissue culture fluids of simian sarcoma virus (SSV) transformants. However, the vast majority remains cell-associated. Thus, this growth factor-related transforming gene product is not a classical secretory protein. These findings define possible cellular locations where the transforming activity of the sis/PDGF-2 protein may be exerted. A scheme for partial purification of biologically active v-sis-coded protein from cells transformed with SSV has made possible a functional comparison of the transforming protein with PDGF. The SSV-transforming gene product is capable of specifically binding PDGF receptors, stimulating tyrosine phosphorylation of PDGF receptors, and inducing DNA synthesis in quiescent fibroblasts. Each of these activities was specifically inhibited by antibodies to different regions of the v-sis gene product. Moreover, viral infection of a variety of cell types revealed a strict correlation between those cells possessing PDGF receptors and those susceptible to transformation by SSV. These findings provide evidence that SSV-transforming activity is mediated by the interaction of a virus-coded mitogen with PDGF receptors.

The polypeptide sequence of the v-sis transforming gene product of SSV can be divided into four regions that are likely to represent structural domains of the protein. Mutations were generated in the SSV nucleotide sequence to assay the extent or function of each of these regions. The results indicate that the helper virus-derived amino terminal sequence, as well as a core region homologous to polypeptide chain 2 of PDGF, are required for the transforming function of the protein. Products of transforming but not nontransforming mutants formed dimer structures conformationally analogous to biologically active PDGF.

The structure of the normal human c-sis/PDGF-2 transcript was determined by a combination of cDNA cloning, nuclease S1 mapping and primer extension. Nucleotide sequence analysis revealed that the 3373-nucleotide c-sis/PDGF-2 mRNA contained only a 723-bp coding sequence for the PDGF-2 precursor polypeptide. The coding sequence was flanked by long 5' (1022 bp) and 3' (1625 bp) untranslated regions. The 5' noncoding region, as well as upstream flanking genomic sequences, contained clusters of specific short repeat sequences. The 3'

noncoding sequence lacked the highly conserved polyadenylation signal. A consensus transcriptional promoter sequence, TATAAA, was identified 24 base pairs upstream of the mRNA start site and an enhancer-like TG element was detected about 180 bp downstream from the site of polyadenylation. These findings identify putative regulatory elements of the c-sis/PDGF-2 gene.

Novel v-erbB-related gene in human carcinoma. The cellular gene encoding the receptor for epidermal growth factor (EGF) has considerable homology to the oncogene of avian erythroblastosis virus. In a human mammary carcinoma, a DNA sequence was identified that is related to v-erbB but amplified in a manner that appeared to distinguish it from the gene for the EGF receptor. Molecular cloning of this DNA segment and nucleotide sequence analysis revealed the presence of two putative exons in a DNA segment whose predicted amino acid sequence was closely related to, but different from, the corresponding sequence of the v-erbB/EGF receptor. Moreover, this DNA segment identified a 5-kb transcript distinct from the transcripts of the EGF receptor gene. Thus, a new member of the tyrosine kinase proto-oncogene family has been identified on the basis of its amplification in a human mammary carcinoma.

A survey of human tumor cell lines for increased PDGF or EGF receptors identified five lines which bind from 6 to 13 times more EGF than normal human fibroblasts. Immunoprecipitation analysis links the elevated binding activity to increased quantities of the EGF receptor protein. EGF receptor gene amplification was detected in 2 of the cell lines. No evidence for EGF receptor gene rearrangements was found at the level of DNA or RNA structure. The results suggest that elevated levels of EGF receptor can be associated with at least three distinct mechanisms. These include gene amplification accompanied by rearrangement, gene amplification without accompanied alteration of mRNA transcripts, and extensive expression without gene amplification.

Isolation of new human oncogene, dbl. DNA of a human diffuse B-cell lymphoma induced an unusual transformed focus on NIH/3T3 cells. The transforming gene was serially transmissible, conferred the neoplastic phenotype to NIH/3T3 cells, and appeared to be larger than 20 kbp by analysis of transfectants for conserved human DNA sequences. From a cosmid recombinant DNA library of a third-cycle transfectant, overlapping clones spanning 80 kbp of cellular DNA were isolated. One clone, which contained a 45-kbp insert comprised entirely of human sequences, was shown to be biologically active, with a specific transforming activity of 650 focus forming units/pmol. By restriction mapping and hybridization analysis, this human transforming gene, designated dbl, was unrelated to any previously reported oncogene.

Abrogation of growth factor dependence by oncogenes. Normal mast cells can be propagated in culture when medium is supplemented with IL-3. We demonstrated that Abelson-MuLV (Ab-MuLV) infection of mast cells eliminates dependence on IL-3 for growth. By contrast, Harvey, BALB, and Moloney MSV, which also productively infect mast cells, are unable to relieve IL-3 dependence. Ab-MuLV-induced IL-3-independent lines express the v-abl-specific transforming protein and have phenotypic characteristics of mast cells. These cells also possess high cloning efficiencies in soft agarose and are tumorigenic in nude mice. In addition, Ab-MuLV induces transplantable mastocytomas in pristane-primed adult mice resistant to lymphoid transformation, defining a new hematopoietic target for malignant transformation by this virus. None of the Ab-MuLV-derived transformants express or secrete detectable levels of IL-3 nor is their growth

inhibited by anti-IL-3 serum. These results argue that Ab-MuLV abrogation of the IL-3 requirement is not due to an autocrine mechanism.

BALB/MK-2 mouse epidermal keratinocytes require epidermal growth factor (EGF) for proliferation, and terminally differentiate in response to high Ca^{++} concentration. Infection with retroviruses containing transforming genes of the src as well as the ras oncogene families leads to rapid loss of EGF-independence. In some cases accompanied by alterations in cellular morphology. The virus-altered cells continue to proliferate in the presence of high levels of extracellular calcium but exhibit alterations in normal keratinocyte terminal differentiation that appear to be specific to the particular oncogene. These alterations bear similarities to abnormalities in differentiation observed in naturally occurring squamous epithelial malignancies.

Human fgr proto-oncogene. The cell-derived domain of Gardner-Rasheed feline sarcoma virus (GR-FeSV) consists of a λ -actin- and a tyrosine-specific protein kinase-encoding sequence designated v-fgr. By utilizing a v-fgr probe, it was possible to detect related sequences present at low copy number in DNAs of a variety of mammalian species and to isolate a human fgr homolog. Comparative studies revealed that this human DNA clone represented all but 200 base pairs of v-fgr. Analysis of human genomic DNA demonstrated that the fgr proto-oncogene was distinct from the cellular homologs of other retrovirus onc genes. In addition, the fgr proto-oncogene was localized to the distal portion of the short arm of human chromosome 1 at p36.1-36.2 by in situ hybridization. Taken together, these findings establish that the fgr proto-oncogene is a unique member of the tyrosine kinase gene family.

Several acute transforming retroviruses encode tyrosine-specific protein kinases which possess structural and functional relationships to cell-surface receptors for certain growth factors. One such tyrosine kinase is encoded by the onc gene, v-fgr, of GR-FeSV. In this study, it was demonstrated that certain lymphomas (but not sarcomas or carcinomas) express fgr-related mRNA. This transcript is detected in Burkitt's lymphoma cell lines naturally infected with EBV, but not in EBV-negative Burkitt's lymphoma cells. Normal umbilical cord or peripheral blood lymphocyte lines established in vitro by EBV infection also contain detectable c-fgr mRNA. Moreover, a 50-fold increase of the steady-state c-fgr mRNA concentration is observed when uninfected Burkitt's lymphoma cell lines are deliberately infected with EBV. These findings demonstrate for the first time the induction of a proto-oncogene in response to infection by a DNA tumor virus.

Identification and oncogenic potential of a novel human sis-like gene. cDNA molecules representing the complete coding sequence of a new human gene have been isolated. This gene, designated c-slk, is a member of the src family of oncogenes. Nucleotide sequence analysis revealed that this gene, termed c-slk, encoded a 537-residue protein which was 86% identical to the chicken proto-oncogene product, p60^{C-SRC}, over a stretch of 191 amino acids at its carboxy terminus. In contrast, only 6% amino acid homology was observed within the amino terminal 82 amino-acid residues of these two proteins. It was possible to activate c-slk as a transforming gene by substituting approximately two-thirds of the c-slk coding sequence for an analogous region of the v-fgr onc gene present in Gardner-Rasheed feline sarcoma virus. The resulting hybrid protein molecule expressed in transformed cells demonstrated protein kinase activity with specificity for tyrosine residues.

Genetic relatedness between intracisternal A particles and other oncovirus genera. Intracisternal A particles represent a major oncovirus genus. By reciprocal hybridization between molecularly cloned A particles and representatives of other oncovirus genera, we established pol gene homology with type B, type D and avian type C viruses. The most extensive homology was with mammalian type D viruses. The transcriptional orientation of the IAP genome was determined, as well as evidence indicating that its pol gene, which is apparently defective, contains coding regions for both reverse transcriptase and endonuclease proteins.

Lentivirus studies. Lentiviruses are a subfamily of retroviruses etiologically associated with arthritis, encephalitis, progressive pneumonia and slow neurological diseases in certain species. Relatively little is known about their genome structure, mechanisms of pathogenesis or evolutionary relationships with other subfamilies of retroviruses. In an effort to better understand the mechanisms by which these viruses induce such a variety of chronic diseases, we have molecularly cloned and physically characterized the genomes of CAEV and equine infectious anemia virus (EIAV). The latter, which bears some morphological similarity to the lentiviruses, has yet to be classified definitively as one. We have determined the nucleotide sequence of a highly conserved region within the CAEV and EIAV pol genes. We demonstrate a much closer relationship of their predicted pol gene products to that of the presumed etiological agent of human acquired immune deficiency syndrome (AIDS) than to those of other retroviruses. Additional pairwise comparisons allowed us to generate an evolutionary tree showing that the pol genes of lentiviruses and oncoviruses have evolved from a common progenitor.

The complete nucleotide sequence of EIAV was determined and its genome organization was shown to be similar to those of other lentiviruses in most respects. The sequence data enabled the construction of vectors for expressing EIAV gene products in bacteria. Preliminary efforts in this regard have been successful.

The EIAV virus was determined to be an excellent model for determining the feasibility of developing a lentivirus vaccine, the ultimate goal being a vaccine against human retroviruses, most notably HTLV-III/LAV and AIDS. In some respects, the production of a vaccine to EIAV may be a more formidable task than the production of an AIDS vaccine. The distribution of EIAV is worldwide, the virus has been with us longer, and Issel and his coworkers have shown antigenic variants occur at an astonishing rate, even within individual horses. Thus, the molecular analysis of EIAV with respect to the requirements needed to develop a vaccine will not only contribute to control of equine infectious anemia of horses, but also will represent an important model for the development of vaccines to human retroviruses.

Enhanced G₂ chromosomal radiosensitivity, deficient DNA repair and susceptibility to cancer.

Visible light irradiation: Apparently normal skin fibroblasts from individuals, each with a genetic disorder predisposing to a high risk of cancer (i.e., ataxia telangiectasia (A-T), Gardner syndrome (GS), Fanconi anemia, Bloom syndrome, xeroderma pigmentosum variant and xeroderma groups C and E) all showed a significantly higher incidence of light-induced chromatid breaks than skin fibroblasts from normal donors if exposed to cool-white fluorescent light

during late S-G₂ phase. However, no consistent difference was observed if exposed during G₁ phase. Xeroderma pigmentosum group A cells did not show the late S-G₂ enhanced chromosomal radiosensitivity. These cells are deficient in repair of DNA damage requiring endonucleolytic incision. This observation suggests that endonuclease incision of DNA is required for chromatid break formation after late S-G₂ light exposure. The responses of these mutant human cells to visible light mimic those observed with low level X-irradiation described previously. In contrast to skin fibroblasts, fetal lung fibroblasts of line IMR-90 showed a significantly higher susceptibility to light-induced chromatid damage. This susceptibility tended to decrease with time in culture to levels comparable to those of skin fibroblasts, an observation suggesting some maturation process in the lung cells such as induction of enzymes to cope with the DNA damage inflicted by light exposure.

Skin fibroblasts from (GS) compared with those from normal donors showed a significantly higher incidence of chromatid gaps and breaks following exposure to low-intensity, cool-white fluorescent light during G₂ phase of the cell cycle. It appears from these findings that the increased incidence of chromatid damage in GS fibroblasts results from deficient repair of DNA single-strand breaks which arise from incomplete nucleotide excision of DNA damage during G₂ phase.

The generation of H₂O₂ and the derivative free hydroxyl radical ([•]OH) in cultures of mouse cells grown in the presence of visible light and ambient oxygen was shown previously to be implicated in chromatid damage. Furthermore, chromosome alterations appear to be associated with the spontaneous neoplastic transformation of mouse cells in culture. An attempt was made in this study to reduce the incidence of chromosomal aberrations and delay or prevent the onset of spontaneous neoplastic transformation of freshly isolated mouse cells, both fibroblasts and epidermal keratinocytes, by adding catalase to the culture medium, shielding the cultures from wavelengths <500 nm and providing a gas phase of 0.1% O₂. These conditions significantly decreased the incidence of chromosomal aberrations in both cell types; in fibroblasts they prevented tumorigenicity in nonirradiated syngeneic mice and increased latent periods for tumor development in X-irradiated mice. The epidermal keratinocytes were particularly resistant to spontaneous neoplastic transformation under all conditions tested. These observations on the protective effect of extracellular catalase suggest that H₂O₂, a normal metabolite, and/or the derivative [•]OH can directly or indirectly produce genetic damage and neoplastic transformation in mouse fibroblasts.

X-irradiation: Apparently normal skin fibroblasts from individuals with familial cancer (f.e., from families with a history of neoplastic disease) were found to exhibit enhanced G₂ phase chromosomal radiosensitivity. This radiosensitivity appears to be associated with both a genetic predisposition to cancer and a malignant neoplastic state. Furthermore, enhanced G₂ phase chromosomal radiosensitivity may provide the basis for an assay to detect genetic susceptibility to cancer.

We have shown increased chromosomal radiosensitivity at the G₂ phase of the cell cycle in fibroblast lines from patients with several cancer predisposing syndromes and from A-T heterozygotes. In this study, we show that this assay can indeed blindly distinguish A-T heterozygotes from nonheterozygotes. When

G₂ chromosomal radiosensitivity is used, the values obtained for A-T heterozygotes overlap with those of A-T homozygotes, rather than with normal values, as often happens with other assays. However, X-ray sensitivity is also high in "normal" fibroblast lines from other individuals genetically predisposed to cancer. This assay to detect the A-T gene should, therefore, be used only within A-T families, when the reason for hypersensitivity is more likely to be true heterozygosity for the A-T gene.

The relationship between tumorigenicity and enhanced chromosomal radiosensitivity during the G₂ cell cycle phase was examined through the use of nontumorigenic human cell hybrids and their nontumorigenic and tumorigenic segregants. The hybrid cells were produced by fusion of a normal and tumor cell. The parental lines, including HeLa and three fibroblast lines, one of skin and two of fetal lung origin, were also examined. The tumorigenic lines, which had cytological features associated with clinical cancer, showed a significantly higher incidence of chromatid breaks and gaps following X-irradiation during G₂ than the normal skin line or the nontumorigenic hybrids. The hybrids and their nontumorigenic subclones had cytological features which are predominantly found with a benign clinical course and had the G₂ chromosomal radiosensitivity more characteristic of the normal parental cells. Like nontumorigenic cells, fetal cells exhibited enhanced G₂ chromosomal radiosensitivity which could be suppressed in fetal x tumor cell hybrids. This observation suggests that the molecular basis for radiosensitivity in fetal cells differs from that of tumor cells. The enhanced G₂ chromosomal radiosensitivity of a tumor cell, which appears to result from deficient DNA repair, is suppressed by fusion with a normal cell. Thus, the radiosensitivity, like tumorigenicity, behaves as a recessive trait. Although a Mendelian analysis is not possible with this material, the segregation of enhanced G₂ chromosomal radiosensitivity with the neoplastic phenotype suggests that the two may be genetically linked.

Evidence is presented that susceptibility to plasmacytomagenesis in the BALB/cAnPt mouse is associated with enhanced chromatid radiosensitivity, resulting from deficient repair of DNA strand breaks during G₂ phase. In the BALB/cAnPt mouse, DNA double-strand breaks are repaired more slowly, leaving broken ends available for chromatid interchanges and subsequent chromosomal translocations. From observations on the F₁ hybrid mice, both susceptibility to plasmacytomagenesis and enhanced G₂ chromatid radiosensitivity appear to behave as recessive traits.

CONTRACT IN SUPPORT OF ALL LABORATORY PROJECTS:

STATE OF CALIFORNIA DEPARTMENT OF HEALTH SERVICES (N01-CP-51001-35)

Title: Breeding and Production of 129/J and NFR Mice and Specified Services

Current Annual Level: \$140,000

Man Years: 3.05

Objectives: To provide in vivo support for four major research efforts within the LCMB: (1) Viral genes involved in leukemogenesis: replication-competent type C virus recombinants generated in tissue culture or constructed in vitro; (2) analysis of target cells for transformation by replication-competent mouse leukemia viruses; (3) the role of host immune response in leukemia virus-induced tumors; and (4) heterotransplantation of human tumor cell-derived lines in athymic nude mice.

Major Contributions: The purpose of this contract is to provide support services for research conducted by LCMB; therefore, a discussion of major contributions will be found in the projects conducted by LCMB.

Proposed Course: This contract has been negotiated to run from October 1, 1984 to September 30, 1989.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE		PROJECT NUMBER
NOTICE OF INTRAMURAL RESEARCH PROJECT		Z01CP04930-15 LCMB
PERIOD COVERED October 1, 1985 to September 30, 1986		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Biology of Natural and Induced Neoplasia		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
PI:	P. Arnstein	Veterinary Director LCMB NCI
Others:	S. A. Aaronson	Chief LCMB NCI
	J. S. Rhim	Research Microbiologist LCMB NCI
	K. C. Robbins	Act. Chief, Mol. Genetics Section LCMB NCI
	J. Pierce	Sr. Staff Fellow LCMB NCI
	A. Eva	Visiting Scientist LCMB NCI
	W. Taylor	Research Biologist LCMB NCI
COOPERATING UNITS (if any) J. Riggs and R. Emmons, CA Dept. Health Services, Berkeley, CA; A. Hackett, Peralta Cancer Inst.; M. Gardner, J. Levy, H. Rubin and M. Stampfer, U. CA, San Francisco; and K. Walen, Children's Hospital Medical Ctr., San Francisco.		
LAB/BRANCH Laboratory of Cellular and Molecular Biology		
SECTION Office of the Chief		
INSTITUTE AND LOCATION NCI, NIH, Bethesda, Maryland 20892		
TOTAL MAN-YEARS: 1.0	PROFESSIONAL: 1.0	OTHER: 0.0
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input checked="" type="checkbox"/> (b) Human tissues <input type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p>In vivo studies on the oncogenes <u>sis</u>, <u>ras</u>, <u>erbB</u> and <u>TGFα</u> are producing needed data on the pathogenesis of tumor induction and cell transformation by these genes. Essential differences among these oncogenes are also being studied in parallel in vitro and in vivo experiments. Genetically engineered type C viruses incorporating each oncogene are usually administered to newborn mice. The <u>ras</u>-containing agents produce extremely early tumors (14 days), whereas the other oncogenes studied to date require several months. In addition, murine cells transfected and transformed in vitro are grafted to adult mice to study the progress of neoplasia characteristic for each oncogene. Human cells transformed by oncogenic viruses with and without chemical cocarcinogens are also tested for characteristic tumorigenicity.</p>		

PROJECT DESCRIPTIONNames, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

P. Arnstein	Veterinary Director	LCMB	NCI
S. A. Aaronson	Chief	LCMB	NCI
J. S. Rhim	Research Microbiologist	LCMB	NCI
K. C. Robbins	Act. Chief, Mol. Genetics Section	LCMB	NCI
J. Pierce	Sr. Staff Fellow	LCMB	NCI
A. Eva	Visiting Scientist	LCMB	NCI
O. Segatto	Visiting Fellow	LCMB	NCI
E. Finzi	Medical Staff Fellow	LCMB	NCI
W. Taylor	Research Biologist	LCMB	NCI

Objectives:

1. To conduct experiments to determine the in vivo biology of available cloned oncogenes and selected genetically mapped tumor viruses. The goal is to correlate specific viral genomic sequences with a particular effect on the host (e.g., in vivo replication, viremia, target cell preference, tumor induction and tumor type induced). The viruses and cloned oncogenes for these studies are selected or especially engineered by molecular biology techniques in vitro.
2. To characterize neoplastic transformation in primate cells and selected nonprimate cultures. To correlate morphologic transformation with transplantability and tumor production in athymic nude mice.
3. To test spontaneous human tumors as well as selected animal specimens for relative xenograft malignancy in athymic nude mice; seek correlations of grafting results with cellular genomes and their translation products, including viruses.

Methods Employed:

1. Viral candidate inocula are furnished by LCMB investigators in coded vials as deep-frozen aliquots or cultures of transformed and control cells. Newborn mice are inoculated at approximately 24 hours of age with the cloned retroviruses by appropriate routes (customarily these are intraperitoneal, subcutaneous, intramuscular or intracerebral). Adult mice are inoculated, usually at two months of age, by similar routes. Cells transformed by or known to incorporate natural or transfected oncogenes are received as viable tissue cultures. They are incubated, scored for morphological evidence of neoplasia and harvested by trypsinization (monolayers) or sedimentation (suspension lines) when sufficiently dense. Viable cell counts are done by the trypan blue stain method and appropriate numbers of viable cells are inoculated into groups of four mice of appropriate strain. For scoring intrinsic malignant potential of the oncogene, athymic nu/nu mice are used. If susceptibility or resistance to cellular immune attack are of interest, euthymic syngeneic mice are also injected for comparison. Doses between 10^3 and 10^7 viable cells are most frequently chosen. A new strain of nude mice, N III, having three immunodeficiency loci (bg/bg, xid/xid and nu/nu) will be available for comparison

testing shortly. This strain is known to be deficient in three important immune reactors, T lymphocytes, B lymphocytes and lymphokine-activated killer (LAK) cells. All inoculated mice are monitored by periodic physical examination and appropriate periodic blood sampling until onset of disease or, in the case of nonpathogenic clones, for the designated holding periods up to the normal life span of the breed of mouse. Tumored mice and controls are exsanguinated, all tissues examined for neoplasia, and appropriate specimens processed for virus isolation, cell culture, microscopic characterization, analysis of tumor DNA and RNA (for onc sequences) and proteins (for tumor-specific translation products). All fatalities are also dissected for histopathological diagnosis.

Studies on the oncogene sis are continuing. Other known or putative oncogenes under study are erbB (Pierce), TGF α (Finzi), ras (Aaronson), P53 and FOS (Segatto) and dbT (Eva).

2. Studies on neoplastic transformation of normal primate cells, in collaboration with Drs. Rhim and Aaronson (Z01CP05060-07 and Z01CP04940-19), involve testing cells at all stages during transformation, from the benign contact-inhibited original through intermediate premalignant growth acceleration, loss of contact inhibition, and ultimately to the morphologically complete conversion to malignant cell phenotype. Wherever possible, the original benign cells, all intermediate transformants and the fully converted line are tested in parallel in athymic nude mice for ability to form neoplasms. The tumor tissue is reestablished in culture to confirm "input" karyology as well as to detect any newly acquired properties attributable to in vivo propagation. It is also examined histopathologically to determine the type of tumor produced.

3. Candidate tissues and cultures derived from spontaneous neoplasms are either grafted directly as a whole tissue implant or established in cell culture and then inoculated after adequate replication in vitro. Some of the materials to be tested are furnished by collaborating NCI investigators and by University of California colleagues. A meaningful xenograft malignancy test usually employs four athymic nude mice given 10^6 to 10^7 viable test cells. If progressive tumors result, they are characterized histopathologically as well as by other criteria as indicated (karyology, antigenicity, virus content).

Major Findings:

1. In vivo effects of oncogenes. (a) Sis oncogenesis. Genetically engineered viruses incorporating the simian v-sis or the human c-sis genes produce delayed-onset sarcomas. In recent tests using high titered recombinant viruses, sarcomas were produced in over 75% of the mice inoculated subcutaneously as neonates. Tumors were detected at 2-6 months (average 4 months). In the newborn group, the euthymic (+/nu) mice were as susceptible as the athymic (nu/nu) nudes. Intracerebral inoculation of the same viruses resulted in 100% fatal brain sarcomas. Controls receiving the "carrier" type C virus (without the sis gene) produced no sarcomas by either route. All tumors were serially transplantable to nu/nu mice, but not to syngeneic +/nu mice.

Normal 3T3 cells transformed by sis transfection rarely produce sarcomas in euthymic syngeneic mice, and only at very high multiplicity (10^7 cells). Tumors are regularly produced in nu/nu mice, but often after incubation periods of one month or longer, in contrast to other oncogenes (see below).

(b) Other oncogenes. Ras seems to be the most malignant gene in the murine test system. Ras-containing viruses given to neonates produce fatal sarcomas within two weeks. Normal 3T3 cells transfected with ras produce sarcomas in adult nu/nu and euthymic mice in one week at 10^6 cells and often with as few as 10^3 cells.

ErbB transfected into 3T3 cells results in transformation almost as malignant as ras, giving rise to sarcomas in less than three weeks after grafting. If administered as a recombinant type C virus into neonates, erbB induces tumors in all inoculated animals, but most frequently after longer incubation than ras (4-6 months), and the tumor types are evenly divided among leukemias, sarcomas and hepatocellular carcinomas. The frequency of the liver cancers is particularly interesting.

TGF α -transfected 3T3s behave similarly to sis-transfectants in that they often produce sarcomas after a one-month delay after allograft.

2. Transformation studies. In collaboration with J. Rhim, it has been shown that human keratinocytes can be transformed to malignancy in a two-step process involving immortalization by Ad 12-SV40 followed by malignant conversion by ras. More recently, malignant conversion of the immortalized keratinocytes was induced without ras, but using the carcinogenic chemicals MNG and 4NQO.

Human amniocytes and fibroblasts are also converted to malignancy using SV40 and ras-containing retrovirus.

3. Human tumor studies. A series of five new spontaneous human tumor lines were tested by inoculation of 10^7 cells per nu/nu mouse. Four were morphologically malignant and produced appropriate tumors. One appeared contact inhibited in vitro and did not produce tumors. It was apparently derived from outgrowth of tumor stroma rather than the malignant component.

Publications:

Rhim, J. S., Fujita, J., Arnstein, P. and Aaronson, S. A.: Neoplastic conversion of human epidermal keratinocytes by adenovirus 12-SV40 virus and chemical carcinogens. Science 232: 385-388, 1986.

Rhim, J. S., Sanford, K. K., Arnstein, P., Fujita, J., Jay, G. and Aaronson, S. A.: Human epithelial cell carcinogenesis: combined action of DNA and RNA tumor viruses produces malignant transformation of primary human epidermal keratinocytes. In Barrett, J. C. and Tennant, R. W. (Eds.): Carcinogenesis. New York, Raven Press, 1985, pp. 57-66.

Rubin, H., Chu, B. M. and Arnstein, P.: Dynamics of tumor growth and cellular adaptation after inoculation into nude mice of varying numbers of transformed 3T3 cells and of readaptation to culture of the tumor cells. Cancer Res. 46: 2027-2034, 1986.

Rubin, H., Hennessey, T. L., Sanui, H., Arnstein, P., Taylor, D. and Chu, B. M.: Inheritance of acquired changes in growth capacity of spontaneously transformed BALB/3T3 cells propagated in mice and in culture. Cancer Res. 45: 2550-2559, 1985.

Whalen, K. H. and Arnstein, P.: Induction of tumorigenesis and chromosomal abnormalities in human amniocytes infected with simian virus 40 and Kirsten sarcoma virus. In Vitro Cell. Dev. Biol. 22: 57-65, 1986.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
 NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
 Z01CP04940-19 LCMB

PERIOD COVERED

October 1, 1985 to September 30, 1986

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Viruses and Transforming Genes in Experimental Oncogenesis and Human Cancer

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	S. A. Aaronson	Chief	LCMB	NCI
Others:	S. R. Tronick	Chief, Gene Structure Section	LCMB	NCI
	K. C. Robbins	Acting Chief, Molecular Genetics Section	LCMB	NCI
	J. H. Pierce	Sr. Staff Fellow	LCMB	NCI
	A. Eva	Visiting Scientist	LCMB	NCI
	C. R. King	Staff Fellow	LCMB	NCI
	J. C. Lacial	Visiting Fellow	LCMB	NCI

COOPERATING UNITS (if any)

U. CA (L. Williams); U. MA Med. Ctr., Worcester (J. Greenberger); U. SC (G. Grotendorst); Weizmann Inst. Sci., Israel (D. Givol); Tel Aviv U., Israel (A. Yaniv; A. Gazit); NIEHS, Res. Triangle Park, NC (M. Anderson; S. Reynolds)

LAB/BRANCH

Laboratory of Cellular and Molecular Biology

SECTION

Molecular Biology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

4.0

PROFESSIONAL:

1.0

OTHER:

3.0

CHECK APPROPRIATE BOX(ES)

- (a) Human subjects (b) Human tissues (c) Neither
 (a1) Minors
 (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The goals of this project are to elucidate the mechanisms of action of tumor viruses and to determine the cellular alterations responsible for naturally occurring malignancies. Topics of present interest include: (1) transforming genes of retroviruses and cancer cells; (2) the biology of endogenous retroviruses; (3) the molecular biology of retrovirus replication and transformation; and (4) the application of knowledge gained from these studies to the search for the causes and mechanisms involved in human neoplastic transformation.

During the past year, our investigations have provided important new insights regarding sis and ras oncogenes. Highlights of sis include: elucidation of the v-sis biosynthetic pathway; demonstration that the v-sis gene product shares with PDGF all the biologic properties attributed to the growth factor; demonstration that v-sis transformation requires interaction of its transforming protein with PDGF receptors; assignment of functional domains of the v-sis transforming protein relative to previously defined structural entities; and definition of the human c-sis/PDGF-2 transcriptional unit, including upstream promoter and regulatory signals.

Structure-function relationship studies of the ras oncogene product have defined domains of the p21-ras protein and have shown that efficient transformation by p21-ras can occur with reduced in vitro GTPase activity. New dominant transforming genes have been isolated from a human diffuse B-cell lymphoma as well as mouse hepatocarcinomas.

PROJECT DESCRIPTIONNames, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

S. A. Aaronson	Chief	LCMB	NCI
S. R. Tronick	Chief, Gene Structure Section	LCMB	NCI
K. C. Robbins	Acting Chief, Molecular Genetics Section	LCMB	NCI
J. Pierce	Sr. Staff Fellow	LCMB	NCI
A. Eva	Visiting Scientist	LCMB	NCI
J. C. Lacal	Visiting Fellow	LCMB	NCI
J. Falco	Medical Staff Fellow	LCMB	NCI
E. Finzi	Medical Staff Fellow	LCMB	NCI
G. Kruh	Medical Staff Fellow	LCMB	NCI
C. R. King	Staff Fellow	LCMB	NCI
M. Kraus	Visiting Fellow	LCMB	NCI
S. K. Srivastava	Visiting Fellow	LCMB	NCI
J. E. Dahlberg	Research Microbiologist	LCMB	NCI

Objectives:

1. To study the mechanisms of action of RNA tumor viruses and transforming genes; and
2. To apply knowledge gained from experimental systems to the search for etiologic agents and mechanisms involved in neoplastic transformation of human cells.

Methods Employed:

Standard and developmental techniques in virology, cell biology, immunology, and molecular biology.

Major Findings:

Ras oncogenes and proto-oncogenes. The availability of molecular clones of the normal and activated alleles of human ras proto-oncogenes made it possible to determine the molecular mechanisms responsible for the malignant conversion of these genes. The genetic lesions responsible for activation of a number of ras oncogenes have been localized to single base changes in their p21 coding sequences. Findings during the past year include the following:

Members of the ras gene family are activated as oncogenes in many different human cancers. To systematically determine the frequency at which such genes might be involved in the neoplastic process affecting a specific target tissue, urothelial cells, a large series of urinary tract tumors were surveyed for ras oncogenes by DNA transfection and by molecular genetic analysis. Harvey (H)-ras oncogenes were detected in 2 of 38 tumors by transfection, molecularly cloned in biologically active form, and shown to contain single base changes at codon 61 leading to substitutions of arginine and leucine, respectively, for glutamine at this position. One additional H-ras oncogene was identified in a bladder carcinoma by restriction polymorphisms at codon 12. In 1 of 21 tumors,

a 40-fold amplification of the Kirsten (Ki)-ras gene was detected. No amplification of other ras genes was detected in any of the tumors analyzed. These findings strengthen the conclusion that codons 12 and 61 are the major "hot spots" of ras oncogene activation and suggest that quantitative alterations in expression due to gene amplification may provide an alternative mechanism for ras gene activation in primary human tumors.

Three human ras family proto-oncogenes, c-Ki-ras-1, c-Ki-ras-2, and N-ras, were mapped to chromosome bands 6p11-12, 12p11.1-12.1, and 1p11-13, respectively, by in situ molecular hybridization. Certain human cancers displayed consistent and specific alterations involving chromosomes 1, 16 and 12. The precise chromosomal localization of ras genes permits evaluation of the possible effect of these chromosome changes on the structure and activities of ras proto-oncogenes in human neoplasia.

Using the NIH/3T3 cell transfection assay, activated cellular oncogenes have been detected in around 10% to 20% of human tumors. From a series of DNA preparations from tissues infiltrated with acute myelogenous leukemia (AML), 50% caused transformation of NIH/3T3 cells. Thus, AML appears to be the human tumor with the highest frequency of oncogenes detected by DNA transfection. In each case, the oncogene involved was N-ras, a member of the ras gene family.

Species- and strain-specific spontaneously occurring tumors have been observed in rodents maintained under normal laboratory conditions. Elucidation of the molecular mechanisms associated with the development of these spontaneous tumors may provide a better understanding of tumor development associated with exposure to chemical carcinogens. In view of the high frequency of oncogene activation shown in rodent tumors induced by known chemical carcinogens, oncogene activation in spontaneous tumors of the B6C3F1 mouse and Fischer 344/N rat was investigated by DNA transfection techniques. A marked difference in the presence of activated oncogenes in spontaneous rat tumors versus spontaneous mouse liver tumors was observed. All rat tumors tested failed to yield activated oncogenes, whereas 30% of mouse hepatocellular adenomas and 77% of hepatocellular carcinomas scored positive by DNA transfection. These transforming genes were identified as activated H-ras gene in all the adenoma transfectants. The two remaining hepatocellular carcinomas contained transforming genes that appear not to be members of the known ras gene family. The B6C3F1 mouse liver system might provide a very sensitive assay not only for assessing the potential of a chemical to activate a cellular proto-oncogene, but also for detecting various classes of proto-oncogenes that are susceptible to mutational activation.

Primary cultures of human epidermal keratinocytes grown in NCTC 168 medium supplemented with horse serum, infected with adeno 12-SV40 hybrid DNA viruses, grew, underwent productive and nonproductive transformation and became established lines. The transformed cells contained SV40 large and small tumor antigens but did not contain adenovirus early region (E1A and E1B) messages. The altered cells were not tumorigenic. Subsequent infection of the "flat" nonproducer cells with Ki-MSV produced striking morphological alterations and induced carcinomas when transplanted into nude mice. Thus, neoplastic transformation of primary human epidermal keratinocytes was demonstrated by the combined action of DNA and RNA tumor viruses. Results establish a multistep process in the progression of human epithelial cells towards malignancy.

The high prevalence of ras oncogenes in human tumors has given increasing impetus to efforts aimed at elucidating the structure and function of their p21 products. To identify functionally important domains of the p21 protein, antibodies were generated against synthetic peptides corresponding to various regions of the protein. Antibodies directed against a synthetic peptide fragment corresponding to amino acid residues 161 to 176 in the carboxy-terminal region of the H-ras-encoded p21 molecule specifically recognized H-ras-encoded p21 proteins. This antibody was also shown to strikingly and specifically inhibit the guanine nucleotide-binding function of the p21 protein. The inability of p21 protein to bind guanine nucleotides was associated with a lack of autophosphorylation or GTPase activities. These studies suggest that a region toward its carboxy terminus is directly or indirectly involved in the guanine nucleotide-binding function of the p21 molecule.

Localization of the epitope recognized by Y13-259, a monoclonal antibody which recognizes a shared epitope of ras p21 proteins, was localized to a small amino acid stretch that was shown not to be directly responsible for the known biochemical functions of the molecule. Instead, this epitope reflects a new domain critical to the role of p21, the ras proto-oncogene product, in the normal pathway leading to DNA synthesis in serum-stimulated cells.

Deletion mutants of the viral Harvey ras oncogene were generated by removing different lengths of the gene from either the amino or carboxy terminus. The deletion mutants, ras p21 expressed in *E. coli*, yielded proteins of approximately 8 kd, 10 kd, 12 kd, 14 kd, 17 kd, 18 kd, 19 kd, and 20 kd. These proteins were utilized to identify epitopes recognized by a series of newly generated monoclonal antibodies as well as some previously reported by Furth and associates in 1982. Monoclonals which inhibited GTP binding, a major biochemical activity of the p21 protein, recognized two major domains. These regions were localized from amino acid 5-69 and 107-164, respectively, and were separated by another stretch from residues 70-106, whose antigenic determinants were not directly involved in GTP binding. Thus, the mapping of epitopes within the p21 molecule recognized by monoclonal antibodies has made it possible to localize important functional domains within the ras p21 molecule.

Deletion of small sequences from the viral H-ras gene have been generated, and resulting ras p21 mutants have been expressed in *E. coli*. Purification of each deleted protein allowed the in vitro characterization of GTP-binding, GTPase, and autokinase activities of the proteins. Microinjection of the highly purified proteins into quiescent NIH/3T3 cells, as well as transfection experiments utilizing an LTR-containing vector, were utilized to analyze the biological activity of the deleted proteins. Two small regions, located at 6-23 and 152-165 residues, were shown to be absolutely required for in vitro and in vivo activities of the ras product. By contrast, the variable region comprising amino acids 165-184 were shown not to be necessary for either in vitro or in vivo activities. Thus, we demonstrated that (1) amino acid sequences at positions 5-23 and 152-165 of p21-ras protein are probably directly involved in the GTP-binding activity, (2) GTP binding is required for the transforming activity of ras proteins and, by extension, for the normal function of the proto-oncogenic product, and (3) the variable region at the C-terminus end of the ras p21 molecule from amino acids 165 to 184 is not required for transformation.

We sought to determine whether decreased *in vitro* GTPase activity is uniformly associated with ras p21 mutants possessing efficient transforming properties. Normal H-ras p21-[Gly¹²-Ala⁵⁹] as well as an H-ras p21-[Gly¹²-Thr⁵⁹] mutant exhibited *in vitro* GTPase activities at least fivefold higher than either H-ras p21-[Lys¹²-Ala⁵⁹] or H-ras p21-[Arg¹²-Thr⁵⁹] mutants. Microinjection of as much as 6×10^6 molecules/cell of bacterially expressed normal H-ras p21 induced no detectable alterations of NIH/3T3 cells. In contrast, inoculation of $4-5 \times 10^5$ molecules/cell of each p21 mutant induced morphologic alterations and stimulated DNA synthesis. Moreover, the transforming activity of each mutant expressed in a eukaryotic vector was similar and at least 100-fold greater than that of the normal H-ras gene. These findings establish that activation of efficient transforming properties by ras p21 proteins can occur by mechanisms not involving reduced *in vitro* GTPase activity.

V-sis oncogene/platelet-derived growth factor (PDGF)-2. The v-sis transforming gene encodes the woolly monkey homolog of human PDGF-2. After its synthesis on membrane-bound polyribosomes, the glycosylated precursor dimerizes in the endoplasmic reticulum and travels through the Golgi apparatus. At the cell periphery, the precursor is processed to yield a dimer structurally analogous to biologically active PDGF. Small amounts of two incompletely processed forms are detectable in tissue culture fluids of simian sarcoma virus (SSV) transformants. However, the vast majority remains cell-associated. Thus, this growth factor-related transforming gene product is not a classical secretory protein. These findings define possible cellular locations where the transforming activity of the *sis*/PDGF-2 protein may be exerted. A scheme for partial purification of biologically active v-sis-coded protein from cells transformed with SSV has made possible a functional comparison of the transforming protein with PDGF. The SSV-transforming gene product is capable of specifically binding PDGF receptors, stimulating tyrosine phosphorylation of PDGF receptors, and inducing DNA synthesis in quiescent fibroblasts. Each of these activities was specifically inhibited by antibodies to different regions of the v-sis gene product. Moreover, viral infection of a variety of cell types revealed a strict correlation between those cells possessing PDGF receptors and those susceptible to transformation by SSV. These findings provide evidence that SSV-transforming activity is mediated by the interaction of a virus-coded mitogen with PDGF receptors.

The polypeptide sequence of the v-sis transforming gene product of SSV can be divided into four regions that are likely to represent structural domains of the protein. Mutations were generated in the SSV nucleotide sequence to assay the extent or function of each of these regions. The results indicate that the helper virus-derived amino terminal sequence, as well as a core region homologous to polypeptide chain 2 of PDGF, are required for the transforming function of the protein. Products of transforming but not nontransforming mutants formed dimer structures conformationally analogous to biologically active PDGF.

The structure of the normal human c-sis/PDGF-2 transcript was determined by a combination of cDNA cloning, nuclease S1 mapping and primer extension. Nucleotide sequence analysis revealed that the 3373-nucleotide c-sis/PDGF-2 mRNA contained only a 723-bp coding sequence for the PDGF-2 precursor polypeptide. The coding sequence was flanked by long 5' (1022 bp) and 3' (1625 bp) untranslated regions. The 5' noncoding region, as well as upstream flanking genomic sequences, contained clusters of specific short repeat sequences. The 3'

noncoding sequence lacked the highly conserved polyadenylation signal. A consensus transcriptional promoter sequence, TATAAA, was identified 24 base pairs upstream of the mRNA start site and an enhancer-like TG element was detected about 180 bp downstream from the site of polyadenylation. These findings identify putative regulatory elements of the *c-sis*/PDGF-2 gene.

A genetic study was undertaken of the *v-sis* oncogene. The *v-sis* transforming protein was found to be divisible into distinct structural domains. Since the *v-sis* oncogene product was found to be similar to a natural growth factor, PDGF-2, the logical next step was a search for growth factor receptor genes. These studies resulted in identification and characterization of four regions of the *sis* transforming protein, including the helper virus *env* gene-encoded amino terminus, its PDGF-2-related region, and regions coded by the cell-derived *v-sis* gene sequence either upstream or downstream of the PDGF-2 coding sequence. Identification of additional members of some proto-oncogene families has emerged from findings of related sequences amplified sufficiently in a particular tumor to allow detection.

A survey of human tumor cell lines for increased PDGF or EGF receptors identified five lines which bind from 6 to 13 times more EGF than normal human fibroblasts. Immunoprecipitation analysis links the elevated binding activity to increased quantities of the EGF receptor protein. EGF receptor gene amplification was detected in two of the cell lines. No evidence for EGF receptor gene rearrangements was found at the level of DNA or RNA structure. The results suggest that elevated levels of EGF receptor can be associated with at least three distinct mechanisms. These include gene amplification accompanied by rearrangement, gene amplification without accompanied alteration of mRNA transcripts, and extensive expression without gene amplification.

Novel *v-erbB*-related gene in human carcinoma. The cellular gene encoding the receptor for epidermal growth factor (EGF) has considerable homology to the oncogene of avian erythroblastosis virus. In a human mammary carcinoma, a DNA sequence was identified that is related to *v-erbB* but amplified in a manner that appeared to distinguish it from the gene for the EGF receptor. Molecular cloning of this DNA segment and nucleotide sequence analysis revealed the presence of two putative exons in a DNA segment whose predicted amino acid sequence was closely related to, but different from, the corresponding sequence of the *v-erbB*/EGF receptor. Moreover, this DNA segment identified a 5-kb transcript distinct from the transcripts of the EGF receptor gene. Thus, a new member of the tyrosine kinase proto-oncogene family has been identified on the basis of its amplification in a human mammary carcinoma.

Abrogation of growth factor dependence by oncogenes. Normal mast cells can be propagated in culture when medium is supplemented with IL-3. We demonstrated that Abelson-MuLV (Ab-MuLV) infection of mast cells eliminates dependence on IL-3 for growth. By contrast, Harvey, BALB, and Moloney MSV, which also productively infect mast cells, are unable to relieve IL-3 dependence. Ab-MuLV-induced IL-3-independent lines express the *v-abl*-specific transforming protein and have phenotypic characteristics of mast cells. These cells also possess high cloning efficiencies in soft agarose and are tumorigenic in nude mice. In addition, Ab-MuLV induces transplantable mastocytomas in pristane-primed adult mice resistant to lymphoid transformation, defining a new hematopoietic target for

malignant transformation by this virus. None of the Ab-MuLV-derived transformants express or secrete detectable levels of IL-3 nor is their growth inhibited by anti-IL-3 serum. These results argue that Ab-MuLV abrogation of the IL-3 requirement is not due to an autocrine mechanism.

BALB/MK-2 mouse epidermal keratinocytes require epidermal growth factor (EGF) for proliferation, and terminally differentiate in response to high Ca^{++} concentration. Infection with retroviruses containing transforming genes of the src as well as the ras oncogene families leads to rapid loss of EGF dependence, in some cases accompanied by alterations in cellular morphology. The virus altered cells continue to proliferate in the presence of high levels of extracellular calcium but exhibit alterations in normal keratinocyte terminal differentiation that appear to be specific to the particular oncogene. These alterations bear similarities to abnormalities in differentiation observed in naturally occurring squamous epithelial malignancies.

Isolation of new human oncogene, dbl. DNA of a human diffuse B-cell lymphoma induced an unusual transformed focus on NIH/3T3 cells. The transforming gene was serially transmissible, conferred the neoplastic phenotype to NIH/3T3 cells, and appeared to be larger than 20 kbp by analysis of transfectants for conserved human DNA sequences. From a cosmid recombinant DNA library of a third-cycle transfectant, overlapping clones spanning 80 kbp of cellular DNA were isolated. One clone, which contained a 45-kbp insert comprised entirely of human sequences, was shown to be biologically active, with a specific transforming activity of 650 focus forming units/pmol. By restriction mapping and hybridization analysis, this human transforming gene, designated dbl, was unrelated to any previously reported oncogene.

Genetic relatedness between intracisternal A particles and other oncovirus genera. Intracisternal A particles represent a major oncovirus genus. By reciprocal hybridization between molecularly cloned A particles and representatives of other oncovirus genera, we established pol gene homology with type B, type D and avian type C viruses. The most extensive homology was with mammalian type D viruses. The transcriptional orientation of the IAP genome was determined, as well as evidence indicating that its pol gene, which is apparently defective, contains coding regions for both reverse transcriptase and endonuclease proteins.

Lentivirus studies. A full length DNA clone of the exogenous retrovirus, Caprine arthritis-encephalitis virus (CAEV) was isolated from high molecular weight DNA of CAEV-infected Himalayan tahr ovary cells. Although other restriction maps of CAEV have been published, this is the first time that the proviral DNA has been cloned. The restriction enzyme map of the clone was determined and found to be identical to that of unintegrated linear CAEV DNA except for the presence of cellular flanking sequences. These findings establish that lentiviruses are able to integrate within the infected host cellular genome. The cloned CAEV genome was shown to contain terminal repeats of approximately 450 base pairs in length, and its restriction enzyme map was oriented with respect to the direction of viral RNA transcription. When the cloned CAEV DNA was used as a molecular probe, it failed to detect related proviral sequences in the genomes of a variety of vertebrate species, including the goat, sheep, horse, mouse, and man. When CAEV DNA was hybridized under relaxed conditions to a variety of cloned DNAs representing different oncoviral genera, homology

to mouse mammary tumor virus (MMTV) was observed, while no homology to avian type C or mammalian type A, C, and D retroviruses was detected. This homology was localized to a region in MMTV corresponding to the 3' end of the gag gene and the 5' end of the pol gene.

Lentiviruses are a subfamily of retroviruses etiologically associated with arthritis, encephalitis, progressive pneumonia and slow neurological diseases in certain species. Relatively little is known about their genome structure, mechanisms of pathogenesis or evolutionary relationships with other subfamilies of retroviruses. In an effort to better understand the mechanisms by which these viruses induce such a variety of chronic diseases, we have molecularly cloned and physically characterized the genomes of CAEV and equine infectious anemia virus (EIAV). The latter, which bears some morphological similarity to the lentiviruses, has yet to be classified definitively as one. We have determined the nucleotide sequence of a highly conserved region within the CAEV and EIAV pol genes. We demonstrate a much closer relationship of their predicted pol gene products to that of the presumed etiological agent of human acquired immune deficiency syndrome (AIDS) than to those of other retroviruses. Additional pairwise comparisons allowed us to generate an evolutionary tree showing that the pol genes of lentiviruses and oncoviruses have evolved from a common progenitor.

EIAV is a retrovirus thought to be most closely related to ovine and caprine lentiviruses because of similar ultrastructural and biochemical properties. Recently we have obtained immunological data which indicates that the major gag protein of EIAV, an important pathogen of horses, shares determinants with the analogous proteins of CAEV and ovine progressive pneumonia virus. Furthermore, we have been able to verify by western blot analysis an earlier report by French workers who showed that EIAV p24 is immunologically related to the virus which causes AIDS. These data, and a recent report indicating that molecularly cloned HTLV-III/LAV can heteroduplex with a clone of visna virus, caused us to compare the molecular features of the AIDS viral genome with those of molecular clones of CAEV and EIAV obtained in this laboratory. Southern blot analysis using relaxed conditions has indicated that EIAV and CAEV share substantial sequence homology with each other and to a more limited extent with a portion of the polymerase region of the MMTV genome. Nucleotide sequence analysis of this highly conserved region of the polymerase genes of CAEV and EIAV has been carried out and the presumptive amino acid sequence compared to those for other genera of retroviruses. We could show by this analysis that the AIDS retroviral isolates are more closely related to lentiviruses than to other retroviral genera, suggesting that lentiviruses and HTLV-III/LAV have evolved from a common progenitor.

The EIAV virus was determined to be an excellent model for determining the feasibility of developing a lentivirus vaccine, the ultimate goal being a vaccine against human retroviruses, most notably HTLV-III/LAV and AIDS. In some respects, the production of a vaccine to EIAV may be a more formidable task than the production of an AIDS vaccine. The distribution of EIAV is worldwide, the virus has been with us longer, and Issel and his coworkers have shown antigenic variants occur at an astonishing rate, even within individual horses. Thus, the molecular analysis of EIAV with respect to the requirements needed to develop a vaccine will not only contribute to control of equine infectious anemia of horses, but also will represent an important model for the development of vaccines to human retroviruses.

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NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01CP04941-14 LCMB

PERIOD COVERED

October 1, 1985 to September 30, 1986

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Biochemical Characterization of Retroviruses and onc Genes

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	S. R. Tronick	Chief, Gene Structure Section	LCMB	NCI
Others:	S. A. Aaronson	Chief	LCMB	NCI
	K. C. Robbins	Chief, Molecular Genetics Section	LCMB	NCI
	J. E. Dahlberg	Research Microbiologist	LCMB	NCI
	G. Vecchio	Expert	LCMB	NCI
	A. Eva	Visiting Scientist	LCMB	NCI
	T. Kawakami	Visiting Fellow	LCMB	NCI
	S.-C. Cheah	Medical Staff Fellow	LCMB	NCI

COOPERATING UNITS (if any)

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LAB/BRANCH

Laboratory of Cellular and Molecular Biology

SECTION

Gene Structure Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

4.0

PROFESSIONAL:

1.0

OTHER:

3.0

CHECK APPROPRIATE BOX(ES)

- (a) Human subjects (b) Human tissues (c) Neither
- (a1) Minors
- (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The c-fgr proto-oncogene was shown to be expressed in human B-cells infected with EBV. This is the first demonstration of the induction of human proto-oncogene expression by a DNA virus. The dbl oncogene isolated by A. Eva was characterized in more detail. Probes for comparing the transforming gene to its normal counterpart were prepared and also used to isolate dbl cDNA and study dbl expression in normal and malignant tissues. In research on animal lentiviruses the regulatory regions (LTR) of the caprine arthritis encephalitis virus were sequenced. The genome of the equine infectious anemia virus (EIAV) was molecularly cloned and its nucleotide sequence determined. Broadly reactive radioimmunoassays for detection of animal lentiviruses were developed. Protein products of the EIAV genome are being expressed in bacteria.

PROJECT DESCRIPTIONNames, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

S. R. Tronick	Chief, Gene Structure Section	LCMB	NCI
S. A. Aaronson	Chief	LCMB	NCI
K. C. Robbins	Chief, Molecular Genetics Section	LCMB	NCI
J. E. Dahlberg	Research Microbiologist	LCMB	NCI
G. Vecchio	Expert	LCMB	NCI
A. Eva	Visiting Scientist	LCMB	NCI
T. Kawakami	Visiting Fellow	LCMB	NCI
S.-C. Cheah	Medical Staff Fellow	LCMB	NCI

Objectives:

The purpose of this project are the following: (1) to biochemically characterize animal lentiviruses in order to understand the mechanisms by which these viruses cause disease; (2) to study the role of endogenous retroviral sequences in normal and neoplastic processes of human cells; and (3) to study the cellular analogues (c-onc) of retroviral transforming genes (v-onc) in order to determine their role in the causation of human cancers and how these genes may participate in normal physiological processes such as growth and differentiation.

Methods Employed:

Standard biochemical techniques for nucleic acid isolation and analysis; assays for enzymes of nucleic acid synthesis and degradation; molecular hybridization techniques to detect and characterize viral genomes; recombinant DNA techniques for the cloning and amplification of retroviral genes; analysis of cloned DNAs by restriction endonuclease mapping, nucleotide sequencing, and electron microscopy.

Major Findings:

The expression of the c-fgr (human) proto-oncogene in human cells was studied by northern blotting and S1 analyses. Certain lymphomas but not carcinomas or sarcomas expressed fgr-related mRNA. The positive cells were from Burkitt's lymphomas (BL) infected with EBV virus. BL cells not harboring EBV genomes were negative. Infection of the latter with EBV induced fgr RNA as did infection of normal umbilical cord or peripheral blood lymphocytes. S1 analysis unambiguously identified the mRNA as being transcribed from the c-fgr locus and not from other closely related members of the tyrosine kinase gene family.

The LTRs of CAEV were subjected to nucleotide sequence analysis. The LTRs were detectably related to those of visna virus, and were found to contain typical signals for the control of viral gene transcription. In addition, potential secondary structures were identified that could influence transcription or translation. A full length clone of the integrated EIAV genome was obtained. Nucleotide sequence analysis and subsequent comparisons provided convincing evidence that human AIDS retroviruses are members of the lentivirus subfamily of retroviruses. The complete nucleotide sequence was determined and its genome organization was shown to be similar to those of other lentiviruses in

most respects. The sequence data enabled the construction of vectors for expressing EIAV gene products in bacteria. Preliminary efforts in this regard have been successful.

The physical map of the dbl oncogene has been determined and portions of its normal homologue isolated. The dbl transcript in transfected cells and in human cells from various sources has been identified by using probes derived from genomic dbl sequences. These same probes have been used to isolate dbl DNAs from transfectants and normal tissues. The chromosomal localization of dbl has been determined.

Publications:

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Tronick, S. R., Popescu, N. C., Cheah, M. S. C., Swan, D. C., Amsbaugh, S. C., Lengel, C. R., DiPaolo, J. A. and Robbins, K. C.: Isolation and chromosomal localization of the human fgf proto-oncogene, a distinct member of the tyrosine kinase gene family. Proc. Natl. Acad. Sci. USA 82: 6595-6599, 1985.

Yaniv, A., Dahlberg, J., Gazit, A., Sherman, L., Chiu, I.-M., Tronick, S. R. and Aaronson, S. A.: Molecular cloning and physical characterization of integrated equine infectious anemia virus: molecular and immunologic evidence of its close relationship to ovine and caprine lentiviruses. Virology. (In Press)

Yaniv, A., Dahlberg, J. E., Tronick, S. R., Chiu, I.-M. and Aaronson, S. A.: Molecular cloning of integrated caprine arthritis-encephalitis virus. Virology 145: 340-345, 1985.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01CP04951-10 LCMB

PERIOD COVERED

October 1, 1985 to September 30, 1986

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Molecular Characterization of Retroviruses

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	J. E. Dahlberg	Research Microbiologist	LCMB	NCI
Others:	M. Wang	Visiting Fellow	LCMB	NCI
	T. Kawakami	Visiting Fellow	LCMB	NCI
	S. R. Tronick	Chief, Gene Structure Section	LCMB	NCI
	S. A. Aaronson	Chief	LCMB	NCI
	S. Broder	Chief	COP	NCI
	H. Mitsuya	Expert	COP	NCI

COOPERATING INSTITUTIONS: Hebrew University (A. Yaniv); Hebrew University (K. Perk); Dept. Pathology, Colorado State University, Fort Collins (J. DeMartini); Dept. Microbiology, Pathology and Parasitology, North Carolina State University (L. Coggins).

LAB/BRANCH

Laboratory of Cellular and Molecular Biology

SECTION

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INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

2.0

PROFESSIONAL:

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OTHER:

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CHECK APPROPRIATE BOX(ES)

- (a) Human subjects (b) Human tissues (c) Neither
- (a1) Minors
- (a2) Interviews

SUMMARY OF WORK (Use stenderd unreduced type. Do not exceed the space provided.)

Lentiviruses of sheep, goats and horses have been shown to represent a genetically distinct group of retroviruses to which the AIDS retroviruses (HTLV-III/LAV) are highly related. Molecular clones of caprine arthritis encephalitis virus (CAEV) and equine infectious anemia virus (EIAV) were shown to be highly homologous in their polymerase genes and less so in their gag genes. Nucleotide sequence analysis of a complete molecular clone of EIAV revealed that the genetic organization of the viral genome is very similar to that of visna and HTLV-III. Current efforts to express EIAV gene products in prokaryotic and eukaryotic vectors and to analyze genetic and antigenic drift in the viral glycoprotein should lead to better diagnostic tests and an understanding of the requirements for a vaccine. At the same time, highly potent inhibitors (2',3'-dideoxynucleosides) of the in vitro replication of lentiviruses have been identified and in vivo testing involving pharmacokinetics, long-term toxicity, and effectiveness in clearing animals of retrovirus are being initiated.

PROJECT DESCRIPTIONNames, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

J. E. Dahlberg	Research Microbiologist	LCMB	NCI
M. Wang	Visiting Fellow	LCMB	NCI
T. Kawakami	Visiting Fellow	LCMB	NCI
S. R. Tronick	Chief, Gene Structure Section	LCMB	NCI
S. A. Aaronson	Chief	LCMB	NCI
S. Broder	Chief	COP	NCI
H. Mitsuya	Expert	COP	NCI

Objectives:

To understand the molecular biology of lentiviruses and other retroviruses associated with slow diseases of domestic animals and man; the use of molecular techniques to analyze mechanisms of pathogenesis and to develop diagnostic and therapeutic reagents useful in monitoring and controlling disease.

Methods Employed:

A wide variety of tissue culture and virological techniques are employed to grow and assay retroviruses. Large scale virus purifications are routinely carried out to provide materials for antibody production, and antigen for ELISA, RIA and western blots. Biochemical procedures routinely used include gel electrophoresis, chromatography, immunoelectrophoresis, immunoblotting, protein purification and ultracentrifugation. Intra- and interspecies radioimmunoassays are employed to detect and analyze immunological and genetic relatedness among retroviral structural proteins.

Major Findings:

Lentiviruses are a group of exogenous retroviruses which cause widespread disease in sheep, goats and horses. Molecular clones of caprine arthritis-encephalitis virus (CAEV) and equine infectious anemia virus (EIAV) have been obtained. The availability of these clones permitted us to show for the first time that the gag and pol genes of EIAV share extensive homology with the corresponding genes of CAEV and that both of these lentiviral genomes are more closely related to the genome of HTLV-III/LAV than to the genomes of other retroviral groups. Understanding of the genetic relationships between these lentiviruses has been extended by the nucleotide sequence determination of the EIAV genome. Analysis of this genome indicates that the genes of EIAV are organized in much the same fashion as for visna and HTLV-III/LAV, although EIAV is about 1 kb shorter. The availability of the EIAV clone has permitted us to construct plasmids expressing the gag precursor and a portion of the polymerase gene in bacteria, and efforts to produce viral glycoprotein are underway. Current emphasis is on the study of antigenic variation in the glycoprotein of EIAV, and the use of glycoprotein-specific probes will facilitate rapid detection and sequence analysis of the env genes of different strains of virus. Analysis of antigenic drift is vital to determining whether a vaccine is feasible, and, in this context, EIAV may represent an important model for AIDS, since HTLV-III/LAV also undergoes rapid genetic drift in its env gene.

In part because it is not clear if retroviral vaccines are achievable, and in part because both seropositive animals and AIDS patients could benefit, considerable effort is being devoted to testing drugs for their ability to inhibit retroviral replication. The reverse transcriptase is a logical target for such intervention, and one class of drugs, dideoxynucleosides, appears to be relatively specific inhibitors of the replication of HTLV-III in human T lymphocytes in vitro. We have tested azidothymidine and a number of 2',3'-dideoxynucleosides for their ability to inhibit the replication of CAEV and EIAV. With the exception of 2',3'-dideoxythymidine, all of the nucleoside analogs tested were extremely potent in at least some of the cell lines used at concentrations that were nontoxic. We were able to show that the type of cell, rather than the particular retrovirus tested, determined whether or not a particular analog was potent. Since it is most likely that the nucleosides must be converted to triphosphates to be effective, the level of appropriate kinases may be the major factor in determining if a drug is inhibitory. The in vitro potency of these compounds suggests that in vivo testing is merited.

Publications:

Chiu, I.-M., Yaniv, A., Dahlberg, J. E., Gazit, A., Skuntz, S., Tronick, S. R. and Aaronson, S. A.: Nucleotide sequence evidence for relationship of AIDS retrovirus to lentiviruses. Nature 317: 366-368, 1985.

Dahlberg, J., Chiu, I.-M., Yaniv, A., Gazit, A., Tronick, S. R. and Aaronson, S. A.: Molecular cloning of equine infectious anemia virus and detection of genetic relatedness to lentiviruses and HTLV-III/LAV. In Proceedings of Conference on Equine Infectious Anemia, Prevention and Control. Buenos Aires, Argentina. (In Press)

Dahlberg, J. E. and Yaniv, A.: The use of an interspecies radioimmunoassay to demonstrate that EIA is a lentivirus. In Tashjian, R. J. and Zarish, D. L. (Eds.): Equine Infectious Anemia. A National Review of Policies, Programs, and Future Objectives. Amarillo, Texas, American Quarter Horse Association, 1985, pp. 128-133.

Sherman, L., Gazit, A., Yaniv, A., Dahlberg, J. E. and Tronick, S. R.: Nucleotide sequence analysis of the long terminal repeat of integrated caprine arthritis-encephalitis virus. Virus Res. (In Press)

Yaniv, A., Dahlberg, J., Gazit, A., Sherman, L., Chiu, I.-M., Tronick, S. R. and Aaronson, S. A.: Molecular cloning and physical characterization of integrated equine infectious anemia virus: molecular and immunologic evidence of its close relationship to ovine and caprine lentiviruses. Virology. (In Press)

Yaniv, A., Dahlberg, J. C., Tronick, S. R., Chiu, I.-M. and Aaronson, S. A.: Molecular cloning of integrated caprine arthritis-encephalitis virus. Virology 145: 340-345, 1985.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
 Z01CP04976-09 LCMB

PERIOD COVERED
 October 1, 1985 to September 30, 1986

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
 Carcinogenesis of Mammalian Cells in Culture

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	K. K. Sanford	Chief, In Vitro Carcinogenesis Section	LCMB	NCI
Others:	J. S. Rhim	Research Microbiologist	LCMB	NCI
	M. Potter	Chief	LG	NCI
	J. H. Robbins	Dermatologist	D	NCI
	K. H. Kraemer	Research Scientist	LMC	NCI
	R. E. Tarone	Mathematical Statistician	BB	NCI
	C. W. Boone	Pathologist	DCPC	NCI

COOPERATING UNITS (if any)
 Howard U. Col. Med. (R. Parshad); U. Calif. Col. Med., Irvine (E. Stanbridge);
 Johns Hopkins U. Sch. Med. (J. K. Frost); Childrens Hosp. L.A. (W. E. Benedict);
 Tel Aviv U. (Y. Shiloh); U. NC (M. Swift); Plaza Del Rio Clinic, AZ (M. H. Greene)

LAB/BRANCH
 Laboratory of Cellular and Molecular Biology

SECTION
 In Vitro Carcinogenesis Section

INSTITUTE AND LOCATION
 NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.0	1.0	2.0

CHECK APPROPRIATE BOX(ES)

(a) Human subjects (b) Human tissues (c) Neither

(a1) Minors

(a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Cultures of skin fibroblasts and peripheral lymphocytes from normal and cancer-prone individuals, as well as neoplastic cells transformed in culture or in vivo, are utilized in evaluating the relationship between radiation-induced chromosomal DNA damage, deficient DNA repair, cancer susceptibility and malignant neoplastic transformation. An increased incidence of chromatid damage after x-irradiation during G-2 phase of the cell cycle is associated with both a predisposition to cancer and malignant transformation and can provide the basis of a test for cancer susceptibility. A genetic basis for this radiosensitivity is indicated from studies with somatic cell hybrids. Further genetic studies are now possible with inbred strains of mice resistant and susceptible to plasmacytoma induction and associated G-2 chromatid radiosensitivity. The chromosomal radiosensitivity appears to result from deficient DNA repair during G-2. Another aspect of this project is to develop a reproducible transformation system with human epidermal keratinocytes as an in vitro model for following the progression of biologic and biochemical changes leading to neoplastic transformation. An associated problem is to identify cytomorphologic changes diagnostic of neoplastic transformation to facilitate transfection and transformation studies.

PROJECT DESCRIPTIONNames, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

K. K. Sanford	Chief, In Vitro Carcinogenesis Section	LCMB	NCI
J. S. Rhim	Research Microbiologist	LCMB	NCI
M. Potter	Chief	LG	NCI
J. H. Robbins	Dermatologist	D	NCI
K. H. Kraemer	Research Scientist	LMC	NCI
R. E. Tarone	Mathematical Statistician	BB	NCI
R. W. Boone	Pathologist	DCPC	NCI

Objectives:

The objective of this project is to elucidate, through cell culture studies, mechanisms of neoplastic transformation in human cells. Current emphasis is on the relationship between radiation-induced chromosomal DNA damage, deficient DNA repair, cancer susceptibility and malignant transformation. Efforts are also directed toward developing a transformation system with human epidermal keratinocytes as an in vitro model of human cell carcinogenesis for characterizing changes in DNA repair capacity during neoplastic transformation. Additionally, efforts are in progress (1) to identify cytomorphologic markers of neoplastic transformation for facilitating transfection and transformation studies, (2) to develop a biochemical correlate of enhanced G₂ chromosomal radiosensitivity, and (3) to identify genetic loci associated with susceptibility to cancer in inbred mouse strains.

Methods Employed:

Chromatid breaks, gaps and interchanges following low-level x-irradiation (25, 50, 100 R) or two-hour exposure to low-intensity fluorescent light (effective wavelength 405 nm in visible range) are quantified in skin fibroblasts or stimulated peripheral lymphocytes following experimental treatment. DNA repair inhibitors, caffeine and cytosine arabinoside are used following irradiation to analyze mechanisms. Since repair of lesions is influenced by the stage of the cell cycle and since the chromatid damage is scored in metaphase cells only, the stage of the cell cycle at the time of irradiation can be experimentally manipulated by varying the interval from treatment to fixation of cells.

Kohn's alkaline elution procedure is being used to compare x-irradiation-induced DNA strand breaks and their repair in cells from normal and cancer-prone individuals. In developing a transformation system with normal epidermal keratinocytes, several approaches to induce transformation, including plasmid transfection and carcinogen treatment, are being used.

Major Findings:

1. In a blind study, we have been able to detect carriers of the gene for ataxia telangiectasia (A-T), an autosomal recessive disease with a striking proneness to lymphoid malignancy. The frequency of A-T gene carriers has been estimated to be 1% in white populations. These heterozygotes seem healthy. However, Swift and his colleagues have demonstrated an excess of

cancer and ischemia in this group and have suggested that as many as 5% of deaths from cancer before the age of 45 may be the result of heterozygosity for the A-T gene. We have demonstrated increased chromosomal radiosensitivity at the G₂ phase of the cell cycle in fibroblast lines from patients with several cancer-predisposing syndromes and from A-T heterozygotes. We now show that this assay can indeed distinguish blindly A-T heterozygotes from nonheterozygotes. The 13 cell lines used could be divided into two groups, low or high x-ray sensitivity. Only cells from healthy donors unrelated to A-T patients (i.e., presumed non-A-T heterozygotes) fell into the low sensitivity range, while both the A-T homozygous and heterozygous lines showed the high x-ray sensitivity. Thus, all the healthy individuals who showed the high sensitivity were correctly identified as A-T heterozygotes.

2. The relationship between tumorigenicity and enhanced chromosomal radiosensitivity during the G₂ cell cycle phase was examined through the use of nontumorigenic human cell hybrids and their nontumorigenic and tumorigenic segregants. The hybrid cells were produced by fusion of a normal and tumor cell. The parental lines, including HeLa and three fibroblast lines, were also examined. The tumorigenic lines, which had cytological features associated with clinical cancer, showed a significantly higher incidence of chromatid breaks and gaps following x-irradiation during G₂ than the normal skin line or the nontumorigenic hybrids. The hybrids and their nontumorigenic subclones had cytological features which are predominantly found with a benign clinical course and had the G₂ chromosomal radiosensitivity more characteristic of the normal parental cells. The enhanced G₂ chromosomal radiosensitivity of a tumor cell, which appears to result from deficient DNA repair, is suppressed by fusion with a normal cell. Thus, the radiosensitivity, like tumorigenicity, behaves as a recessive trait. Although a Mendelian analysis is not possible with this material, the segregation of enhanced G₂ chromosomal radiosensitivity with the neoplastic phenotype suggests that the two may be genetically linked.
3. Genetic differences in susceptibility to plasmacytoma induction among inbred strains of mice are well documented. Whether induced by intraperitoneal implantation of plastic discs or injection of pristane, the BALB/c mouse, in contrast to most other commonly used inbred strains, is significantly more susceptible. The F₁ hybrid between BALB/c and a resistant strain such as DBA/2 is also resistant. Mechanisms underlying this susceptibility are largely unknown. However, our recent studies on human cells in culture in relation to cancer susceptibility have provided an approach for exploring this problem. We have obtained evidence using stimulated B lymphocytes and skin fibroblasts that susceptibility to plasmacytomagenesis in the BALB/cAn mouse is associated with enhanced chromatid radiosensitivity, resulting from deficient repair of DNA strand breaks during G₂ phase. From observations on the F₁ hybrid mice, both susceptibility to plasmacytomagenesis and enhanced G₂ chromatid radiosensitivity appear to behave as recessive traits. Furthermore, the deficiency in DNA repair in B lymphocytes of the BALB/cAn mouse may contribute to the formation of the rcpt 12:15 and rcpt 6:15 chromosomal translocations that occur in over 98% of pristane-induced plasmacytomas. These translocations deregulate or activate the c-myc oncogene, confer a strong proliferative stimulus and apparently play a critical role in plasmacytomagenesis.

Publications:

- Boone, C. W., Sanford, K. K., Frost, J. K., Mantel, N., Gill, G. W. and Jones, G. M.: Cytomorphologic evaluation of the neoplastic potential of 28 cell culture lines by a panel of diagnostic cytopathologists. Int. J. Cancer. (In Press)
- Jones, G. M., Sanford, K. K., Parshad, R., Gantt, R., Price, F. M. and Tarone, R. E.: Influence of added catalase on chromosome stability and neoplastic transformation of mouse cells in culture. Brit. J. Cancer 52: 583-590, 1985.
- Parshad, R., Sanford, K. K. and Jones, G. M.: Chromatid damage induced by fluorescent light during G₂ phase in normal and Gardner syndrome fibroblasts. Interpretation in terms of deficient DNA repair. Mutat. Res. 151: 57-63, 1985.
- Parshad, R., Sanford, K. K. and Jones, G. M.: Chromosomal radiosensitivity during the G₂ cell cycle period of skin fibroblasts from individuals with familial cancer. Proc. Natl. Acad. Sci. USA 82: 5400-5403, 1985.
- Sanford, K. K., Parshad, R. and Gantt, R.: Enhanced G₂ chromosomal radiosensitivity, susceptibility to cancer and neoplastic transformation. In Skamene, E. (Ed.): Genetic Control of Host Resistance to Infection and Malignancy. New York, Alan R. Liss, Inc., 1985, pp. 811-820.
- Sanford, K. K., Parshad, R. and Gantt, R.: Responses of human cells in culture to hydrogen peroxide and related free radicals generated by visible light: relationship to cancer susceptibility. In Johnson, J. E., Jr. (Ed.): Free Radicals, Aging and Degenerative Diseases. New York, Alan R. Liss, Inc. (In Press)
- Sanford, K. K., Parshad, R., Potter, M., Nordan, R. P., Brust, S. E. and Price, F. M.: Chromosomal radiosensitivity during G₂ phase and susceptibility to plasmacytoma induction in mice. In Potter, M. and Melchers, F. (Eds.): Current Topics in Microbiology and Immunology. Mechanisms in B-cell Neoplasia. New York, Springer-Verlag. (In Press)
- Sanford, K. K., Parshad, R., Stanbridge, E. J., Frost, J. K., Jones, G. M., Wilkinson, J. E. and Tarone, R. E.: Chromosomal radiosensitivity during the G₂ cell cycle period and cytopathology of human normal x tumor cell hybrids. Cancer Res. 46: 2045-2049, 1986.
- Shiloh, Y., Parshad, R., Sanford, K. K. and Jones, G. M.: Carrier detection in ataxia-telangiectasia. Lancet 1: 689-690, 1986.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01CP05060-08 LCMB

PERIOD COVERED

October 1, 1985 to September 30, 1986

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Studies of Oncogenic Expression in Animal and Human Cancers

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	J. S. Rhim	Research Microbiologist	LCMB	NCI
Others:	S. A. Aaronson	Chief	LCMB	NCI
	J. B. Park	Visiting Fellow	LCMB	NCI
	P. Arnstein	Veterinary Director	LCMB	NCI
	K. Sanford	Chief, In Vitro Carcinogenesis Section	LCMB	NCI
	D. Ablashi	Research Microbiologist	LCMB	NCI
	J. E. Dahlberg	Research Microbiologist	LCMB	NCI

COOPERATING UNITS (if any)

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NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

2.5

PROFESSIONAL:

1.0

OTHER:

1.5

CHECK APPROPRIATE BOXES)

- (a) Human subjects (b) Human tissues (c) Neither
- (a1) Minors
- (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The goals of this project are: (1) to establish and define a cell culture transformation system for identification of carcinogenic agents and humans at high risk for cancer; (2) to develop human cell transformation systems, with particular emphasis on epithelial cells, in order to study host factors regulating cell transformation and the mechanisms of carcinogenesis by chemicals, viruses, hormones and x-irradiation; (3) to isolate and characterize oncogenes from human or primate tumors; and (4) to develop and test measures to prevent and/or control cell transformation and the neoplastic event for eventual clinical application.

Major findings were: (1) establishment of a nontumorigenic human epidermal keratinocyte line immortalized by exposure to Ad 12-SV40 virus; (2) malignant transformation of human epidermal keratinocytes by the combined action of Ad 12-SV40 virus and Ki-MSV; (3) neoplastic conversion of human keratinocytes by Ad 12-SV40 virus and chemical carcinogens (MNNG or 4NQO), which should be useful in assessing environmental carcinogens and detecting new human oncogenes; (4) keratin expression of tumorigenic chemically and virally transformed human epidermal cells was similar to that of control cells; (5) thyroid hormone optimized transformation by Ki-MSV; (6) hydrocortisone enhanced expression of Epstein-Barr virus (EBV) genomes in human cells; (7) Ha-ras was found to be activated in about 10% of human urinary tract tumors; (8) an N-ras gene was detected in 7060 human rectal carcinoma-derived cells, as well as a restriction fragment length polymorphism of human c-myb gene.

PROJECT DESCRIPTIONNames, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

J. S. Rhim	Research Microbiologist	LCMB	NCI
S. A. Aaronson	Chief	LCMB	NCI
J. B. Park	Visiting Fellow	LCMB	NCI
P. Arnstein	Veterinary Director	LCMB	NCI
K. Sanford	Chief, In Vitro Carcinogenesis Section	LCMB	NCI
D. V. Ablashi	Research Microbiologist	LCMB	NCI
J. E. Dahlberg	Research Microbiologist	LCMB	NCI

Objectives:

1. To develop sensitive in vitro transformation assays to identify carcinogenic agents and humans at high risk for cancer.
2. To develop and study human cell transformation systems, particularly epithelial cells, and the factors regulating cell transformation to elucidate mechanisms of cell transformation by carcinogenic agents and viruses.
3. To search for human and primate oncogenes from human and primate cancers.
4. To develop measures to prevent and/or control cell transformation and cancer in animals and ultimately in humans.

Methods Employed:

Biological methods include cell and virus cloning, transformation focus, cell aggregation, soft agar, genome rescue and DNA-mediated gene transfer (DNA transfection) using NIH/3T3 cells as recipients to detect activated cellular transforming genes in malignant tumors. A number of biochemical and molecular biological techniques, including gel electrophoresis, Southern blotting, immunoprecipitation and restriction endonuclease analysis are used to characterize the activated oncogenes. Gene cloning into phage and plasmid is used for characterization of oncogenes.

Major Findings:Neoplastic transformation of human epidermal keratinocytes by Ad 12-SV40 and Kirsten murine sarcoma viruses.

Eighty percent of human cancers are thought to arise from epithelial cells. Thus, an epithelial cell culture system is a crucial tool for studying the genetic and cellular changes that take place in cells during malignant transformation and the ways in which carcinogens and oncogenes induce the process.

Primary human epidermal keratinocytes acquired indefinite life span in culture but did not undergo malignant conversion in response to infection with a hybrid or Ad 12-SV40. The Ad 12-SV40-altered cells contained both SV40 large and small tumor antigens, but did not contain adenovirus early region (E1A and E1B)

messages. Subsequent addition of Ki-MSV, which contains a K-ras oncogene, to these cells induced striking morphological alterations associated with the acquisition of neoplastic properties. These findings demonstrate the malignant transformation of human primary epithelial cells in culture by the combined action of Ad 12-SV40 and Ki-MSV and support a multiple-step process for neoplastic conversion.

Neoplastic conversion of human keratinocytes by Ad 12-SV40 virus and chemical carcinogens. The established nontumorigenic human epidermal line immortalized by exposure to Ad 12-SV40 virus was transformed by treatment with the chemical carcinogens MNNG and 4NQO. Such transformants showed morphological alterations, including tumorigenicity in nude mice, whereas primary human epidermal keratinocytes treated with these carcinogens failed to show any evidence of transformation. These findings demonstrate the malignant transformation of human epithelial cells in culture after exposure to the virus-carcinogen combination. This system will be useful in evaluating the carcinogenic potential of environmental chemicals and for studying what genes are activated and suppressed in the multistep process leading to malignancy.

Keratin expression of both chemically- and virally-transformed human epidermal keratinocytes during the process of neoplastic conversion. Human epidermal cells, despite being "immortalized" or "transformed" by combinations of either oncogenic virus (SV40, Ad 12 or Ki-MSV) or chemical carcinogen (MNNG or 4NQO), exhibited keratins similar (although quantitatively reduced) to that of control cells when grown in vitro. However, athymic nude mouse tumors derived from such cells exhibited suppression of the 52-, 56- and 58-kd (basic type II) keratins and a predominance of small-sized (40-48 or 50 kd, acidic type I) keratins. The synthesis of these specific keratins was resumed following re-establishment of cell lines in culture. These results suggest that the changes in keratin protein profiles frequently exhibited by human carcinomas represent a component of the pleomorphic transformed phenotype which can be uncoupled from neoplastic growth.

Thyroid hormone modulation of transformation induced by Ki-MSV. We have investigated the effect of triiodothyronine (T_3) on the transformation of normal rat kidney (NRK) cells by Ki-MSV. The results indicated that thyroid hormone is a required factor for optimal transformation by Ki-MSV and that the hormone exerts its effects during the early phase of Ki-MSV-induced transformation.

Hydrocortisone enhancement of both EBV replication and transformation of human cord lymphocytes. We investigated the interaction of hydrocortisone and EBV. The treatment of P3HR-1 cells (propagated at 34°C and 37°C) with various concentrations of hydrocortisone for 7 and 21 days resulted in enhanced levels of antigen-positive cells with a maximum increase at 21 days. Virus harvested from hydrocortisone-treated P3HR-1 cells grown at 34°C had a 1-2 log higher titer in Raji cells when compared to control virus. These data suggest that hydrocortisone is able to enhance the expression of the EBV genomes present in human cells and leads to increased levels of antigen expression and virus production. The mechanism by which this glucocorticoid hormone modulates EBV expression remains to be determined.

Frequency of molecular alterations affecting ras proto-oncogenes in human urinary tract tumors. We surveyed a large series of urinary tract tumors for ras oncogenes by DNA transfection and by molecular genetic analysis. Harvey (Ha)-ras oncogenes were detected in 2 of 38 tumors by transfection and shown to contain single-base changes at codon 61 leading to substitutions of arginine and leucine, respectively, for glutamine at this position. One additional Ha-ras oncogene was identified in a bladder carcinoma by restriction polymorphisms at codon 12. In one of 21 tumors, we observed a 40-fold amplification of the K-ras gene. No amplification of other ras genes was detected in any of the tumors analyzed. Our findings suggest that quantitative alterations in expression due to gene amplification may provide an alternative mechanism for ras gene activation in primary human tumors.

An activated N-ras oncogene in 7060 human rectal carcinoma-derived cells and a restriction fragment length polymorphism of the c-myb gene. An N-ras transforming gene was detected in 7060 human rectal carcinoma-derived cells and molecularly cloned. An Eco RI restriction fragment length polymorphism, consisting of two alleles, of the human c-myb gene was detected. This suggests the relationship between the phenotype changes in the c-myb locus and the induction of the 7060 tumor.

Publications:

Ablashi, D. V., Whitman, J., Dahlberg, J., Armstrong, G. and Rhim, J. S.: Hydrocortisone enhancement of both EBV replication and transformation of human cord lymphocytes. In Levine, P. H., Ablashi, D. V., Pearson, G. R. and Kottaridis, S. D. (Eds.): Developments in Medical Virology. EBV and Associated Diseases. Hingham, Massachusetts, Martinus Nijhoff Publishing Co., 1985, pp. 392-401.

Banks-Schlegel, S. and Rhim, J. S.: Keratin expression by both chemically- and virally-transformed human epidermal keratinocytes during the process of neoplastic conversion. Carcinogenesis 7: 153-157, 1986.

Borek, C., Ong, A. and Rhim, J. S.: Thyroid hormone modulation of transformation induced by Kirsten murine sarcoma virus. Cancer Res. 45: 1702-1706, 1985.

Fujita, J., Srivastava, S. K., Kraus, M. H., Rhim, J. S., Tronick, S. R. and Aaronson, S. A.: Frequency of molecular alterations affecting ras proto-oncogenes in human urinary tract tumors. Proc. Natl. Acad. Sci. USA 82: 3849-3853, 1985.

Rhim, J. S., Fujita, J., Arnstein, P. and Aaronson, S. A.: Neoplastic conversion of human epidermal keratinocytes by adenovirus 12-SV40 virus and chemical carcinogens. Science 232: 385-388, 1986.

Rhim, J. S., Jay, G., Arnstein, P., Price, F. M., Sanford, K. K. and Aaronson, S. A.: Neoplastic transformation of human epidermal keratinocytes by Ad 12-SV40 and Kirsten sarcoma viruses. Science 227: 1250-1252, 1985.

Rhim, J. S., Sanford, K. K., Arnstein, P., Fujita, J., Jay, G. and Aaronson, S. A.: Human epithelial cell carcinogenesis: Combined action of DNA and RNA tumor viruses produces malignant transformation of primary human epidermal keratinocytes. In Barrett, J. C. and Tennant, R. W. (Eds.): Carcinogenesis Vol. 9. New York, Raven Press, 1985, pp. 57-66.

Yuasa, Y., Reddy, E. P., Rhim, J. S., Tronick, S. R. and Aaronson, S. A.: An activated N-ras oncogene in 7060 human rectal carcinoma-derived cells and a restriction fragment length polymorphism of the c-myb gene. Jap. J. Cancer Res. (In Press)

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01CP05062-08 LCMB

PERIOD COVERED

October 1, 1985 to September 30, 1986

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Transforming Genes of Naturally-Occurring and Chemically-Induced Tumors

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	A. Eva	Visiting Scientist	LCMB	NCI
Others:	S. A. Aaronson	Chief	LCMB	NCI
	S. R. Tronick	Chief, Gene Structure Section	LCMB	NCI
	G. C. Vecchio	Expert	LCMB	NCI
	S. K. Srivastava	Visiting Fellow	LCMB	NCI
	D. Ron	Visiting Fellow	LCMB	NCI
	L. Varesio	Visiting Scientist	LMI	NCI
	J. Ward	Chief, TPCS	LCC	NCI

COOPERATING UNITS (if any)

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Molecular Biology Section

INSTITUTE AND LOCATION

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TOTAL MAN-YEARS:

1.5

PROFESSIONAL:

1.0

OTHER:

0.5

CHECK APPROPRIATE BOX(ES)

- (a) Human subjects (b) Human tissues (c) Neither
- (a1) Minors
- (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

A human transforming gene, dbl, was isolated from the DNA of a primary human diffuse B-cell lymphoma by the DNA transfection assay on NIH/3T3 cells. This human oncogene was cloned in a biologically active form in a cosmid vector as a human DNA sequence of 4.5 kilobases. By hybridization studies between cellular and viral oncogenes and their cloned human transforming gene, dbl was shown to be unrelated to any previously reported oncogenes. A cDNA library was constructed in λ GT11 with polyadenylated RNA purified from a dbl third-cycle transfectant. Several clones were isolated and their structure and nucleotide sequence are now being analyzed.

Using sera obtained from mice bearing tumors induced by dbl transfectant, a 66,000 dalton protein (p66) was specifically detected in dbl-transformed NIH/3T3 cells. This protein was found to be the translational product of dbl oncogene. Moreover, preliminary subcellular localization studies reveal that p66 was distributed between cytosol and crude membrane fraction, p66 could be phosphorylated in vivo, and phosphoserine appears to be the only detectable phosphoamino acid residue on p66 protein.

We had earlier analyzed, by transfection assay, DNAs of two groups of MCA-induced fibrosarcomas in BALB and NIH Swiss mice for their ability to transform NIH/3T3 cells and found that an activated K-ras was detectable in 50% of the tumor DNAs analyzed. Cell lines were established from one of the two groups of tumors and analyzed for their growth capability in vivo. Results obtained so far seem to indicate that the presence of an activated K-ras gene is associated with strikingly faster growth of the tumor cells in vivo as compared with the growth of MCA tumor cells that do not contain an activated ras gene.

PROJECT DESCRIPTIONNames, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

A. Eva	Visiting Scientist	LCMB	NCI
S. A. Aaronson	Chief	LCMB	NCI
S. R. Tronick	Chief, Gene Structure Section	LCMB	NCI
G. C. Vecchio	Expert	LCMB	NCI
S. K. Srivastava	Visiting Fellow	LCMB	NCI
D. Ron	Visiting Fellow	LCMB	NCI
L. Varesio	Visiting Scientist	LMI	NCI
J. Ward	Chief, Tumor Pathol. & Pathog. Sec.	LCC	NCI
W. McBride	Chief, Cellular Regulation Sec.	LB	NCI

Objectives:

Studies are directed to identify transforming genes associated with specific human hematopoietic malignancies. Isolation and characterization of these genes are pursued in order to determine their mechanisms of activation and their specific involvement in the human malignant process.

Methods Employed:

Standard and developmental techniques in cell biology, biochemistry and recombinant DNA are used in these studies.

Major Findings:

The dbl oncogene was cloned in a biologically active form in a cosmid vector. A series of fragments free of human repetitive sequences were isolated from the cosmid clone and used to probe northern blots of mRNAs isolated from the transfectant cell lines. Several of the probes detected a 2.9-kb fragment expressed in the transfectants but not in control NIH/3T3 cells. The detection of the dbl gene transcript allowed us to construct a cDNA library in λ GT11 with a dbl transfectant polyadenylated RNA. The library was screened with one of the probes derived from the cloned gene and several dbl-specific cDNA clones were identified and isolated. One of them, a 1.8-kb cDNA fragment, was completely sequenced. The nucleotide sequence analysis of the dbl cDNA clone failed to reveal homology with any oncogene product or other sequences available in the gene bank data base. We were also able to identify the product of this transforming gene utilizing antisera raised against dbl-transformed cells as a 66,000 dalton protein specific to dbl transfectants. The p66 protein was found to be a phosphoprotein whose major site of phosphorylation is on serine residues.

Cell lines have been established from several MCA-induced fibrosarcomas. High molecular weight DNA was extracted from each of these cell lines and analyzed for transforming activity on NIH/3T3 cells. Fifty percent of the DNA analyzed was found to contain an activated K-ras gene. These cell lines were injected into nude mice and assessed for their malignant potential. We observed that injection of 10^4 cells from each of the cell lines containing an activated K-ras oncogene resulted in the growth of fibrosarcomas in 100% of the tested animals within 4 weeks after inoculation. Moreover, up to 80% of the animals

died of the tumor within 10 weeks after inoculation. On the other hand, only 80% of the animals inoculated with 10^4 cells from the cell lines that did not contain an activated K-ras gene developed tumors and the cumulative mortality was significantly lower, 70% of the animals being alive 10 weeks after inoculation. Finally, the tumor cell growth was significantly different in the two groups of animals. The mean tumor size of the group injected with cells without detectable K-ras oncogene was always about 50% of that of mice injected with MCA tumor cells containing an activated K-ras gene.

Publications:

Eva, A. and Aaronson, S. A.: Isolation of a new human oncogene from a diffuse B-cell lymphoma. Nature 316: 273-275, 1985.

Eva, A., Aaronson, S. A., and Tronick, S. R.: Transforming genes of human malignancies. Proceedings of the 18th Jerusalem Symposium. (In press)

Eva, A., Pierce, J. and Aaronson, S.A.: Interactions of oncogenes with hematopoietic cells. In Gale, R. P. and Golde, D. W. (Eds.): Leukemia: Recent Advances in Biology and Treatment. New York, Alan R. Liss, Inc., 1985, pp. 3-15.

Pierce, J., Eva, A. and Aaronson, S. A.: Hematopoietic cell targets for transformation by retroviral oncogenes. In Peschle, C. and Rizzoli, C. (Eds.): New Trends in Experimental Hematology. Rome, Ares Serono Symposia, 1984, pp. 1-18.

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NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01CP05063-08 LCMB

PERIOD COVERED

October 1, 1985 to September 30, 1986

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Studies on Epstein-Barr Virus and HTLV-III

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	D. V. Ablashi	Research Microbiologist	LCMB	NCI
Others:	S. Z. Salahuddin	Expert	LTCB	NCI
	R. C. Gallo	Chief	LTCB	NCI
	A. G. Palestine	Chief, Clin. Immunol. Section	LI	NEI
	R. B. Nussenblatt	Deputy Clinical Director	CB	NEI

COOPERATING INSTITUTES: Wistar Institute, Philadelphia, PA; Johns Hopkins University, Baltimore, MD; University of Pennsylvania, Philadelphia, PA; University of Maryland, Baltimore, MD; Johns Hopkins University, Baltimore, MD; Wistar Institute, Philadelphia, PA; S. M. Gravelly, College of Medicine, Howard University, Washington, DC; R. D. Markham, Litton Bionetics, Kensington, MD

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INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20982

TOTAL MAN-YEARS:

1.0

PROFESSIONAL:

1.0

OTHER:

0.0

CHECK APPROPRIATE BOX(ES)

- (a) Human subjects (b) Human tissues (c) Neither
 (a1) Minors
 (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Epstein-Barr virus (EBV) associated with B-cell lymphomas was recently isolated from the plasma of AIDS patients in our laboratory. In view of this development and recent reports of a 30-fold increase in B-cell lymphomas in AIDS patients, we attempted to investigate infection and replication of HTLV-III in B-cells. We found that only (but not all) EBV genome-positive cells could be infected by HTLV-III, whether or not they possessed T4 receptors. Two EBV-positive lines became chronically infected with HTLV-III. Three EBV genome-negative cell lines (lacking T4 receptors) could not be infected with HTLV-III until they were converted to EBV positivity. Even after infection, however, the latter remained negative for T4 receptors, suggesting that the mechanism of infection of B-cells by HTLV-III does not involve T4 antigens. Alternatively, EBV may induce a receptor that may be required for HTLV-III infection. Results suggest the interaction of EBV and HTLV-III in B-cell lymphomas associated with AIDS.

The specific role of megakaryocytes is not fully known except that they are involved in platelet formation. Three human megakaryocyte cell lines were found to contain the EBV genome. One of these lines also possessed approximately 3% EBV virus capsid antigens (VCA). This was the first report of the ability of megakaryocytes to replicate EBV, suggesting that this cell type possesses EBV receptors. Like the EBV genome-positive B-cells, the megakaryocyte lines were infectible with HTLV-III, further supporting the role of EBV in HTLV-III infection and adding another cell type for HTLV-III replication.

PROJECT DESCRIPTIONNames, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

D. V. Ablashi	Research Microbiologist	LCMB	NCI
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R. C. Gallo	Chief	LTCB	NCI
A. G. Palestine	Chief, Clin. Immunol. Section	LI	NEI
R. B. Nussenblatt	Deputy Clinical Director	CB	NEI

Objectives:

1. Investigation of the in vitro interaction of Epstein-Barr virus (EBV) and HTLV-III which could explain the etiology of B-cell lymphomas in AIDS.
2. Based on our reported observations of detectable EBV genomes in 70% of megakaryocyte cell lines, studies were conducted to determine EBV infectivity of human megakaryocyte cell lines derived from normal individuals, one patient with acute lymphocytic lymphoma, and one patient with autoimmune neutropenia.
3. Malaria has been implicated in the etiology of Burkitt's lymphoma (BL) in Africa. EBV is present in most BL cells from endemic areas and African children with EBV antibody titers are at greater risk of developing BL. To study the role of malaria in BL, we studied the expression of EBV early antigen (EA) in BL cell lines after exposure to *Plasmodium falciparum*-infected red blood cells (IRBC) and also the rate of DNA synthesis in treated cells.
4. After our initial isolation of HTLV-III from the tears of an AIDS patient, we investigated the frequency of virus isolation, transmission of cell-free virus and identification of cell type(s) in which the virus replicates. This is important for understanding the mechanism of replication of HTLV-III in ocular cells which, in turn, should provide measures to prevent spread of infection during routine ophthalmologic examinations and corneal transplants.

Methods Employed:

Primary cell cultures and continuous human B- and T-cell lines, with and without the EBV genome, were used for HTLV-III infection and measurement of EBV and HTLV-III antigens using monoclonal antibodies. Tears and ocular tissues were collected from AIDS patients and healthy individuals for HTLV-III detection. Primary ocular tissues after separation into epithelial, endothelial and stroma cells were cultured in vitro for HTLV-III infection and morphological identification. Standard biochemical and immunological procedures were applied. *P. falciparum* was cultured in human type O, Rh-positive red blood cells (RBC) and synchronized according to standard procedures. Normal RBCs were maintained in the same manner. Schizont-IRBCs were isolated from supernatant medium after centrifugation and were stored at -20° C until used. Human EBV genome-positive genome-negative cell lines, after infection with *P. falciparum*, were used to detect EBV antigens by use of monoclonal antibodies to EA and VCA, as well as human sera possessing EBV antibodies. DNA synthesis in treated and control cultures was measured after incorporation of ³H-thymidine.

Major Findings:

1. Five out of 10 EBV genome-positive cell lines could be infected with HTLV-III regardless of the presence of T4 antigen. None of the EBV genome-negative B cell lines (Bjab, Ramos and Louks) could be infected with HTLV-III. However, the latter were infectible after conversion to EBV-positive status, suggesting the induction of a receptor by EBV that may be similar to the T4 receptor required for HTLV-III infection. The virus recovered from these cell lines was infectious for human cord blood mononuclear cells. Infection of EBV EBNA-positive cell lines with HTLV-III did not result in induction of EBV-EA. Only one out of three EBV virus-producer cell lines got infected with HTLV-III, and both EBV and HTLV-III particles were observed in these cells. The supernatants of this dually infected cell culture contained more EBV. It appears that the T4 receptor is not essential for HTLV-III infection of B-cells and that the presence of EBV may be the only requirement for infection of such cells, although it is not 100%. The above findings could lead to a better understanding of the role of these cells in HTLV-III-related diseases and may also contribute to understanding the development of B cell lymphomas frequently observed in HTLV-III-infected individuals.

2. Three human megakaryocyte cell lines (SAL, EVA, CAT) originated from acute lymphocytic leukemia (SAL), autoimmune neutropenia (EVA) and a normal donor (CAT) were found to contain EBV nuclear antigen (EBNA). Only SAL expressed >3% EBV-VCA. Besides the B lymphoblastoid cells, only the epithelial cells from nasopharyngeal carcinoma (NPC) have been found to contain EBV, suggesting a very narrow cell specificity. Thus, this is the first evidence that EBV can infect and establish a latent genome in megakaryocytes. It is likely that EBV may be responsible for continuous growth of these cells. After treatment of these cell lines with a tumor promoter (TPA), EA was inducible (>5-10%). Both types of EA (R and D) could be detected by monoclonal antibodies as well as human EBV EA-positive sera. The SAL cell line, after 72-hour treatment with TPA, showed typical herpesvirus particles in various stages of replication, providing further evidence of EBV replication.

Like B-cell lines expressing EBNA, all three megakaryocyte cell lines were infectible with HTLV-III as early as 10 days postinfection. Infected cells expressed reverse transcriptase activity (RT) and 1-3% expressed HTLV-III, P15 and P24 antigens, as detected by indirect immunofluorescence (IF). Typical HTLV-III particles were also observed. The cell-free supernatant from these cell lines contained infectious HTLV-III. The specific effect of the interaction of EBV and HTLV-III in these cells is unclear. However, megakaryocytes lacking T4 receptors can replicate HTLV-III. Thus, this adds another cell type that is infectible with HTLV-III.

3. B-cell line (Raji), established from African BL, contains the EBV genome and does not produce any EBV antigen. Our results showed that *P. falciparum* IRBC and NRBC induced expression of the latent EBV genome (EBV-EA). The induction of EA-R and EA-D varied. However, there were >9-16% EA-positive cells after 72 hours of treatment with *P. falciparum*-infected RBC, and <3% EA-positive cells were also detected with NRBC-treated Raji cells. None of the EA-positive cells went on to demonstrate VCA. The data show that induction of EA by *P. falciparum* is similar to that observed in Raji cells treated with TPA. However, induction is much earlier in megakaryocytes and the percentage of positive cells is also higher.

A significant increase in DNA synthesis in Raji cells was also observed. Other B-cell lines positive for EBNA lacked any increased DNA synthesis. Some DNA synthesis was also caused by NRBBC. Since EA induction does not require DNA synthesis, however, EBV-DNA in Raji cells replicates with host cell DNA. These data provide indirect evidence that malaria is a contributing factor in the etiology of BL in endemic areas. Induction of EA by malaria in EBV genome-positive cells is the first evidence of its kind.

4. Nine out of 14 (64.3%) tear samples from AIDS patients expressed HTLV-III when these specimens were used to infect human cord blood mononuclear cells. Tears from seven healthy normals contained no HTLV-III. There was considerable variability in the amount of HTLV-III expressed. Cell-free supernatants from 2/9 HTLV-III tear sample-infected human cord blood mononuclear cells contained transmissible HTLV-III. Infected cultures contained HTLV-III particles and HTLV-III P24- and P15-positive cells, as detected by IF. Only half (33.3%) of the AIDS patients positive for HTLV-III by RT showed 2-5% P24/P15-positive cells from the conjunctival scrapings. Conjunctival cells from normal healthy donors were negative for HTLV-III antigens. One asymptomatic HTLV-III antibody-positive individual and one AIDS patient's corneal cells contained HTLV-III. HTLV-III antigen-positive cells were observed in cultured cornea epithelium. Weak antigen-containing cells were found in cultured endothelial cells. No antigen was found in fibroblast cells from the cornea. Although no documented cases of AIDS have been reported in cornea transplant recipients, serologic screening of donors prior to the use of tissue for transplantation would appear advisable. Our data also raise important questions regarding possible transmission of HTLV-III during ophthalmologic examination by way of the examiner's hands, through instruments and during contact lens fittings.

Publications:

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Ablashi, D. V., Schirm, S., Fleckenstein, B., Faggioni, A., Dahlberg, J., Rabin, H., Loeb, W., Armstrong, G., Whang-Peng, J., Aulakh, G. and Torrisi, M. R.: Herpesvirus saimiri-induced lymphoblastoid rabbit cell line: growth characteristics, virus persistence and oncogenic properties. J. Virol. 55: 623-633, 1985.

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Levine, P. H., Ablashi, D. V., Pearson, G. R. and Kottaridis, S. D. (Eds.): Developments in Medical Virology: EBV and Associated Diseases. Hingham, Massachusetts, Martinus Nijhoff Publishing Co., 1985, pp. 1-693.

Morgan, D. and Ablashi, D. V.: Detection of EBNA and rescue of transforming EBV in megakaryocyte cells established in culture. In Levine, P. H., Ablashi, D. V., Pearson, G. R. and Kottaridis, S. D. (Eds.): Developments in Medical Virology: EBV and Associated Diseases. Hingham, Massachusetts, Martinus Nijhoff Publishing Co., 1985, pp. 392-401.

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NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01CP05164-06 LCMB

PERIOD COVERED

October 1, 1985 to September 30, 1986

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Interaction of Hematopoietic Cells and Mammalian Retroviruses

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	J. H. Pierce	Sr. Staff Fellow	LCMB	NCI
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Others:	S. A. Aaronson	Chief	LCMB	NCI
	P. Di Fiore	Visiting Fellow	LCMB	NCI
	J. Falco	Medical Staff Fellow	LCMB	NCI
	M. Kraus	Visiting Fellow	LCMB	NCI

COOPERATING UNITS (if any)

University of Virginia, Charlottesville (J. T. Parsons); University of Massachusetts Medical Center, Worcester (J. Greenberger)

LAB/BRANCH

Laboratory of Cellular and Molecular Biology

SECTION

Molecular Biology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

2.0

PROFESSIONAL:

1.0

OTHER:

1.0

CHECK APPROPRIATE BOX(ES)

- (a) Human subjects (b) Human tissues (c) Neither
- (a1) Minors
- (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

(1) A recombinant murine retrovirus vector containing the v-erbB gene of avian erythroblastosis virus was generated and transformed a variety of established mammalian cell lines. The murine erbB virus was also capable of abrogating the EGF-dependence of murine epithelial cells and induced pre-B lymphoid transformation in a murine bone marrow colony-forming assay. (2) To more clearly define the characteristics of hematopoietic cell populations transformed by ras, src, fes and abl oncogenes, continuous clonal cell lines established in our laboratory were examined for phenotypic and functional markers specific to various hematopoietic lineages. The majority of transformed cell lines, irrespective of the oncogene-containing retrovirus used for their induction, possessed characteristics of immature B lymphocytes. However, some ras-induced transformants coexpressed early B lymphoid and myeloid antigens and could be manipulated to differentiate along either pathway. These results suggest that a multipotential progenitor cell may exist which is a target for ras-induced transformation and supports the hypothesis of a close developmental relationship between B lymphoid and myeloid lineages. (3) A long-term interleukin-3 (IL-3)-dependent basophilic cell line was utilized to investigate the mechanisms involved in conversion to a factor-independent neoplastic state. Abrogation of IL-3 dependence occurred spontaneously at very low frequency and at a very high frequency after infection with tyrosine kinase-coding retroviral oncogenes. In all cases, release of factor dependence correlated with conversion of the cells from a nontumorigenic to a tumorigenic phenotype. The mechanisms by which oncogene-induced and spontaneous acquisition of factor independence occur are currently being investigated.

PROJECT DESCRIPTIONNames, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

J. H. Pierce	Sr. Staff Fellow	LCMB	NCI
S. A. Aaronson	Chief	LCMB	NCI
P. Di Fiore	Visiting Fellow	LCMB	NCI
J. Falco	Medical Staff Fellow	LCMB	NCI
M. Kraus	Visiting Fellow	LCMB	NCI
K. Holmes	Staff Fellow	LIP	NCI
H. Morse	Chief	LIP	NCI
W. Davidson	Visiting Associate	LG	NCI

Objectives:

To analyze the biological and biochemical properties of murine recombinant retroviruses generated in our laboratory which contain growth factor receptor-related genes.

To analyze immature murine hematopoietic cell lines transformed by a variety of retroviruses in this laboratory for unique phenotypic characteristics in order to provide a means for identifying functions involved in the early stages of hematopoietic cell differentiation.

To investigate and compare the mechanisms by which normal growth factor-dependent hematopoietic cells are converted to factor-independent malignant cells either by oncogene induction or as a spontaneous event.

Methods Employed:

Standard hematopoietic culture techniques included an in vitro hematopoietic colony-forming assay developed to detect transformation of hematopoietic cells by retroviruses, use of feeder layers to establish continuous cell lines, and cloning of established cell lines in soft agar. Other procedures included factor-dependent colony-forming assays, generation of growth factors and retrovirus infection of factor-dependent cell lines.

Identification of hematopoietic phenotype of retrovirus-transformed hematopoietic cells was performed utilizing histochemical staining, immunofluorescence techniques, radioimmunoassays and enzymatic assays.

Identification of retrovirus-specific transforming oncogenes in hematopoietic transformants was performed by Southern and northern hybridization analysis, radioimmunoprecipitation and immunofluorescence techniques.

Major Findings:

A recombinant murine retrovirus vector containing the v-erbB gene of avian erythroblastosis virus was constructed in order to investigate v-erbB as a transforming gene for mammalian cells. The construct, designated MuLV/erbB, transformed NIH/3T3 cells at a high efficiency in the DNA transfection assay. Individual MuLV/erbB transfectants grew in soft agar and were tumorigenic.

The transfectants contained v-erbB DNA sequences, expressed v-erbB-specific transcripts, and synthesized v-erbB-related glycoproteins. The majority of transfectants produced two major v-erbB gene products of 58 and 66 kd. However, some transfectants produced much smaller v-erbB-specific proteins. These findings indicate that expression of the complete v-erbB gene product is not required for transformation of NIH/3T3 cells.

A transmissible murine erbB (M-erbB) virus was generated by infection of non-producer transfectants with amphotropic murine leukemia virus. The M-erbB virus was shown to induce morphological transformation of a variety of established cells lines, including rat kidney, mink epithelial and human fibroblasts. In addition, M-erbB virus abrogated the epidermal growth factor requirements of a murine epithelial cell line. We also demonstrated that M-erbB virus transformed immature hematopoietic cells both in vitro and in vivo. M-erbB virus-induced hematopoietic colonies could be established in culture as continuous clonal lines with high proliferative capacity. Their ability to form rapidly growing tumors of donor origin confirmed their malignant potential. The hematopoietic blast cell transformants induced by M-erbB virus did not express phenotypic markers associated with cells within the erythroid lineage. However, all in vitro- and in vivo-derived lines analyzed possessed several characteristics of early cells within the B lymphoid lineage. Moreover, the pathology of the M-erbB virus-induced disease in mice was strikingly similar to that observed after Abelson-MuLV infection. Analysis of M-erbB virus-transformed hematopoietic cells revealed that they did not express detectable levels of EGF receptors. These findings support the concept that the range of target cells whose growth can be altered by an oncogene that possesses homology with growth factor receptor is not limited to cells that utilized that particular growth factor regulatory pathway for proliferation.

Previous studies in our laboratory have revealed that several oncogene-containing retroviruses are capable of transforming murine hematopoietic cells by utilizing a colony-forming assay that is dependent upon the presence of 2-mercaptoethanol. To more clearly define the characteristics of the cell populations transformed by *ras*, *src*, *fes* and *abl* oncogenes, continuous clonal cell lines produced from infected bone marrow and fetal liver cultures were examined for expression of lineage-specific cell-surface antigens, expression of cytoplasmic μ chain, and the organization of immunoglobulin heavy and light chain genes. The results showed that cell lines with characteristics of pro-B and pre-B cells were obtained from cells infected with viruses containing *bas*, *H-ras*, *K-ras* and *src*, as well as *abl* and *fes*. In addition, three cell lines transformed by *ras*-containing viruses were found to coexpress antigens usually restricted to the B cell or myeloid pathways of differentiation. Detailed analyses of these lines suggest that the initial transforming event giving rise to these lines occurred in a precursor common to the B cell and myeloid lineages. These studies indicate that an unexpectedly wide range of onc genes can induce B-lineage lymphomas in vitro. The availability of cell lines with both myeloid and B cell differentiation potentials provides a unique opportunity to explore the molecular and biochemical events that define irrevocable commitment to these distinct hematopoietic lineages.

We have demonstrated that IL-3-dependent cells can be abrogated from dependence by infection with Abelson-MuLV without evidence of autocrine stimulation. To further investigate the mechanism by which factor independence and neoplastic conversion occurs, we analyzed a long-term IL-3-dependent basophilic line, 32D. Several factor-independent variants of this line were obtained at a high frequency by infection with oncogene-containing retroviruses of the tyrosine kinase family. In addition, spontaneous factor-independent variants were isolated at a low frequency. In all cases, the acquisition of factor independence correlated with conversion of the cells from a nontumorigenic to a tumorigenic phenotype. We are currently analyzing the spontaneously transformed variants to determine whether they are releasing IL-3 and become autonomous via an autocrine mechanism. In addition, parameters such as increased tyrosine phosphorylation, cellular oncogene expression and IL-3 gene rearrangement are being investigated in the spontaneous variants in comparison to the original IL-3-dependent line and the oncogene-abrogated transformants.

Publications:

Eva, A., Pierce, J. and Aaronson, S. A.: Interactions of oncogenes with hematopoietic cells. In Gale, R. P. and Golde, D. W. (Eds.): Leukemia: Recent Advances in Biology and Treatment. New York, Alan R. Liss, Inc., 1985, pp. 3-15.

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Naparstek, E., Pierce, J. H., Metcalf, D., Shadduck, R., Ihle, J., Leder, A., Sakakeeny, M. A., Wagner, K., Falco, J. and Greenberger, J. S.: Induction of growth alterations in factor-dependent hematopoietic progenitor cell lines by co-cultivation with irradiated bone marrow stromal cell lines. Blood. (In Press)

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Pierce, J. H., Eva, A. and Aaronson, S. A.: Interactions of oncogenes with hematopoietic cells. In Gale, R. P. and Hoffbrand, V. (Eds.): Acute Leukaemia. London, W. B. Saunders Ltd. (In Press)

Pierce, J. H., Gazit, A., Di Fiore, P. P., Kraus, M., Pennington, C. Y., Holmes, K. L., Davidson, W. F., Mores, H. C., III and Aaronson, S. A.: Mammalian cell transformation by a recombinant murine retrovirus containing the avian erythroblastosis virus erbB gene. In Potter, M. (Ed.): Current Topics in Microbiology and Immunology. Berlin/Heidelberg, Springer-Verlag. (In Press)

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Robbins, K. C., Leal, F., Pierce, J. H., and Aaronson, S. A.: The v-sis/PDGF-2 transforming gene product localizes to cell membranes but is not a secretory protein. EMBO J. 4: 1783-1792, 1985.

NOTICE OF INTRAMURAL RESEARCH PROJECT

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October 1, 1985 to September 30, 1986

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Mechanisms of Transformation Induced by Retrovirus onc Genes

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	S. R. Tronick	Chief, Gene Structure Section	LCMB	NCI
	T. Miki, N. Giese	Guest Researchers	LCMB	NCI
	S. Katamine	Visiting Fellow	LCMB	NCI
	M. Cheah	Medical Staff Fellow	LCMB	NCI
	T. Kawakami	Visiting Fellow	LCMB	NCI
	T. Fleming	Guest Researcher	LCMB	NCI

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INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

3.0

PROFESSIONAL:

1.0

OTHER:

2.0

CHECK APPROPRIATE BOX(ES)

- (a) Human subjects (b) Human tissues (c) Neither
- (a1) Minors
- (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Our findings have shown that the v-sis translational product is functionally equivalent to platelet-derived growth factor (PDGF). Findings that v-sis was able to induce transformation only of cell types responsive to the proliferative action of PDGF demonstrated that the PDGF-like activities of the v-sis translational product were the functions responsible for its transforming ability. We have also shown that transcriptional activation of the normal human v-sis/PDGF-2 gene in cells capable of responding to PDGF leads to morphologic transformation of such cells. We have defined the full extent of the sis transcriptional unit. Promoter signals were identified immediately upstream of the mRNA start site in normal human genomic DNA. Identification and isolation of these putative regulatory sequences make it now possible to investigate the biologic consequences of c-sis/PDGF-2 gene transcriptional activation in normal human cells.

A survey of human tumor cells for the presence of fgr proto-oncogene mRNA has revealed that expression of this gene is highly restricted. Normal or transformed B-lymphocytes naturally or deliberately infected with Epstein-Barr virus expressed detectable c-fgr mRNA. These and other findings demonstrated for the first time induction of a proto-oncogene in response to infection by a DNA tumor virus. The normal human fgr gene has been isolated. Human fgr DNA clones made it possible to demonstrate that the fgr proto-oncogene is distinct from cellular homologues of other tyrosine kinase-encoding onc genes. The fgr proto-oncogene was localized to the distal portion of the short arm of human chromosome 1 at p36.1 by in situ hybridization. Taken together, our findings establish that the fgr proto-oncogene is a unique member of the tyrosine kinase gene family.

PROJECT DESCRIPTIONNames, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

K. C. Robbins	Act. Chief, Molecular Genetics Section	LCMB	NCI
S. A. Aaronson	Chief	LCMB	NCI
S. R. Tronick	Chief, Gene Structure Section	LCMB	NCI
T. Miki	Guest Researcher	LCMB	NCI
S. Katamine	Visiting Fellow	LCMB	NCI
M. Cheah	Medical Staff Fellow	LCMB	NCI
T. Kawakami	Visiting Fellow	LCMB	NCI
N. Giese	Guest Researcher	LCMB	NCI
T. Fleming	Guest Researcher	LCMB	NCI

Objectives:

1. To define the interaction of the simian sarcoma virus (SSV)-transforming protein (p28^{sis}) with the platelet-derived growth factor (PDGF) receptor.
2. To assess the role of the sis-proto-oncogene in human neoplasia.
3. To determine the mechanism of Gardner-Rasheed feline sarcoma virus-induced oncogenesis.
4. To elucidate the function of the normal human fgr-proto-oncogene.

Methods Employed:

Standard biochemical techniques for nucleic acid isolation and analysis; assays for enzymes of nucleic acid synthesis and degradation; radioimmunoassays for qualitative and quantitative characterization of gene products; in vitro synthesis and immunoprecipitation; peptide synthesis; antibody production; fractionation of cellular components and protein purification to analyze gene products; molecular hybridization techniques to analyze genes; recombinant DNA techniques for the purification and amplification of genes; analysis of genetic structure using restriction endonuclease mapping, nucleotide sequencing, and electron microscopy techniques; DNA transfection and construction of virus mutants for analysis of transforming activity.

Major Findings:

The structure of the normal human c-sis/platelet-derived growth factor 2 (sis/PDGF-2) transcript was determined by a combination of cDNA cloning, nuclease S_I mapping, and primer extension. Nucleotide sequence analysis revealed that the 3373-nucleotide sis/PDGF-2 mRNA contained only a 723-base-pair (bp). Coding sequence was flanked by long 5' (1022 bp) and 3' (1625 bp) untranslated regions. The 5' noncoding region, as well as upstream flanking genomic sequences, contained clusters of specific short repeat sequences. A consensus transcriptional promoter sequence, TATAAA, was identified 24 bp upstream of the mRNA start site and an enhancer-like "TG element" was detected about 180 bp downstream from the site of polyadenylation. These findings identify putative regulatory elements of the sis/PDGF-2 gene.

A scheme for partial purification of biologically active v-sis-coded protein from cells transformed with simian sarcoma virus (SSV) has made possible a functional comparison of the transforming protein with platelet-derived growth factor (PDGF). The SSV-transforming gene product is capable of specifically binding PDGF receptors, stimulating tyrosine phosphorylation of PDGF receptors, and inducing DNA synthesis in quiescent fibroblasts. Each of these activities was specifically inhibited by antibodies to different regions of the v-sis gene product. Moreover, viral infection of a variety of cell types revealed a strict correlation between those cells possessing PDGF receptors and those susceptible to transformation by SSV. These findings provide evidence that SSV-transforming activity is mediated by the interaction of a virus-coded mitogen with PDGF receptors.

The cell-derived domain of Gardner-Rasheed feline sarcoma virus (GR-FeSV) consists of a γ -actin and a tyrosine-specific protein kinase coding sequence, designated v-fgr. Utilizing a v-fgr probe, it was possible to detect related sequences present at low copy number in DNAs of a variety of mammalian species and to isolate a human fgr homologue. Comparative studies revealed that this human DNA clone represented all but 200 base pairs of v-fgr. Analysis of human genomic DNA demonstrated that the fgr proto-oncogene was distinct from the cellular homologues of other retrovirus onc genes. In addition, the fgr proto-oncogene was localized to the distal portion of the short arm of human chromosome 1 at p36.1 by *in situ* hybridization. Taken together, our findings establish that the fgr proto-oncogene is a unique member of the tyrosine kinase gene family.

The primary translation product of GR-FeSV consists of helper virus-coded p15 sequences, a portion of γ -actin and a tyrosine-specific protein kinase. The GR-FeSV tyrosine kinase is closely related in amino acid sequence to the products of avian v-yes and v-src genes but is derived from a distinct proto-oncogene. Utilizing DNA probes which represent the GR-FeSV tyrosine kinase gene, v-fgr, we have surveyed human tumor cells for expression of the fgr proto-oncogene. Transcripts related to v-fgr were detected in several lymphoid tumor cell lines but only in a few of the sarcomas or carcinomas examined. A single transcript, 3 kb in length, was detected in approximately 50% of the Burkitt's lymphomas examined.

Further analysis showed that American Burkitt's lymphomas were uniformly negative, whereas the fgr proto-oncogene was transcriptionally active in all African Burkitt's tested. It was of interest that expression correlated with the presence of the Epstein-Barr virus (EBV) genome in all the Burkitt's lymphomas analyzed. To examine this association further, cord and peripheral lymphocytes immortalized by EBV infection were shown to express the 3-kb fgr-related transcript. American Burkitt's lymphoma cell lines infected with either prototype B95-8 EBV or the P3HR1 mutant, which is incapable of immortalizing peripheral lymphocytes, were shown to express the fgr proto-oncogene transcript. All of these findings taken together demonstrate that EBV infection of lymphocytes induces expression of the fgr proto-oncogene. It is not known whether the function absent from the P3HR1 mutant is alone sufficient for EBV-induced immortalization. Thus, the establishment function of EBV may involve cooperation between activities coded by this herpesvirus and the fgr proto-oncogene.

Publications:

Aaronson, S. A., Robbins, K. C. and Tronick, S. R.: The role of proto-oncogenes in normal growth and neoplasia. Proceedings of XVII Congresso Della Societa Italiana di Patologia. Florence, Italy, 1984. (In Press)

Aaronson, S. A., Tronick, S. R. and Robbins, R. C.: Oncogenes and pathways to malignancy. In Boynton, A. L. and Leffert, H. L. (Eds.): Control of Animal Cell Proliferation. New York, Academic Press, 1985, pp. 3-24.

Cheah, M. S. C., Igarashi, H., Leal, F., Naharro, F. and Robbins, K. C.: Growth factor mediated proliferative pathways and the neoplastic process. Cancer Investigation. (In Press)

Cheah, M. S. C., Ley, T. J., Tronick, S. R. and Robbins, K. C.: fgr proto-oncogene mRNA induced in B lymphocytes by Epstein-Barr virus infection. Nature 319: 238-240, 1986.

Cheah, M. S. C., Ley, T. J., Tronick, S. R. and Robbins, K. C.: Induction of fgr proto-oncogene RNA in B lymphocytes as a consequence of Epstein-Barr virus infection. In Potter, M. (Ed.): Current Topics in Microbiology and Immunology. Berlin, Springer-Verlag. (In Press)

Igarashi, H., Gazit, A., Chiu, I.-M., Srinivasan, A., Yaniv, A., Tronick, S. R., Robbins, K. C. and Aaronson, S. A.: Normal human sis/PDGF-2 gene expression induces cellular transformation. In Feramisco, J., Ozanne, B. and Stiles, C. (Eds.): Cancer Cells 3/Growth Factors and Transformation. New York, Cold Spring Harbor Laboratories, 1985, pp. 159-166.

Kawakami, T., Cheah, M. S. C., Leal, F., Igarashi, H., Pennington, C. Y. and Robbins, K. C.: Involvement of polypeptide growth factors and their receptors in the neoplastic process. In Valeriote, F., Crissman, J. and Al-Sarraf, M. (Eds.): Head and Neck Cancer: Scientific Principles and Management. Amsterdam, Elsevier. (In Press)

King, C. R., Giese, N. A., Kraus, M. H., Robbins, K. C. and Aaronson, S. A.: Oncogenes as growth factors and growth factor receptors: genetic studies of v-sis and a novel erbB-related gene. In Galeotti, T., Citadini, A., Neri, G., Papa, S. and Smets, L. A. (Eds.): Cell Membranes and Cancer. Amsterdam, Elsevier, 1985, pp. 411-416.

King, C. R., Giese, N., Robbins, K. C. and Aaronson, S. A.: In vitro mutagenesis of the v-sis transforming gene defines functional domains of its PDGF-related product. Proc. Natl. Acad. Sci. USA 82: 5295-5299, 1985.

Leal, F., Igarashi, H., Gazit, A., Williams, L. T., Notario, V., Tronick, S. R., Robbins, K. C. and Aaronson, S. A.: Mechanism of transformation by an oncogene coding for a normal growth factor. Proc. UCLA Symposia on Molecular & Cellular Biology. Biochemical and Molecular Epidemiology of Cancer. (In Press)

Leal, F., William, L. T., Robbins, K. C. and Aaronson, S. A.: Evidence that the v-sis gene product transforms by interaction with the receptor for platelet-derived growth factor. Science 230: 327-330, 1985.

- Rao, C. D., Igarashi, H., Chiu, I.-M., Robbins, K. C. and Aaronson, S. A.: Structure and sequence of the human c-sis/PDGF-2 transcriptional unit. Proc. Natl. Acad. Sci. USA 83: 2392-2396, 1986.
- Robbins, K. C. and Aaronson, S. A.: Elucidation of a normal function for a human proto-oncogene. In Luderer, A. A. and Westall, H. H. (Eds.): Molecular Analysis and Diagnosis of Malignancy. New Jersey, Humana Press, Inc. (In Press)
- Robbins, K. C., Igarashi, H., Gazit, A., Chiu, I.-M., Tronick, S. R. and Aaronson, S. A.: Establishing the link between human proto-oncogenes and growth factors. In Castellani, A. (Ed.): Epidemiology and Quantitation of Environmental Risk in Humans from Radiation and Other Agents. New York, Plenum Press, 1985, pp. 459-470.
- Robbins, K. C., King, C. R., Giese, N. A., Leal, F., Igarashi, H. and Aaronson, S. A.: Involvement of oncogene-coded growth factors in the neoplastic process. In Papas, T. S. and Vande Woude, G. G. (Eds.): Gene Amplification and Analysis. Oncogenes and Growth Factors. Amsterdam, Elsevier. (In Press)
- Robbins, K. C., Leal, F., Pierce, J. H. and Aaronson, S. A.: The v-sis/PDGF-2 transforming gene product localizes to cell membranes but is not a secretory protein. EMBO J. 4: 1783-1792, 1985.
- Tronick, S. R., Popescu, N. C., Cheah, M. S. C., Swan, D. C., Amsbaugh, S. C., Lengel, C. R., DiPaolo, J. A., and Robbins, K. C.: Isolation and chromosomal localization of the human fgr proto-oncogene, a distinct member of the tyrosine kinase gene family. Proc. Natl. Acad. Sci. USA 82: 6595-6599, 1985.
- Tronick, S. R., Yuasa, Y., Robbins, K. C., Eva, A., Gol, R. and Aaronson, S. A.: Oncogene research: closing in on a better understanding of cancer causation. In Selikoff, I. J., Teirstein, A. S. and Hirschman, S. Z. (Eds.): Acquired Immune Deficiency Syndrome. Annals of the New York Academy of Sciences. New York, The New York Academy of Sciences, 1984, pp. 150-160.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01CP05234-05 LCMB

PERIOD COVERED

October 1, 1985 to September 30, 1986

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Molecular Cloning and Characterization of Transforming Genes

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: V. Notario Visiting Associate LCMB NCI

Others: K. C. Robbins Act. Chief, Molecular Genetics Section LCMB NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Cellular and Molecular Biology

SECTION

Molecular Biology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

1.0

PROFESSIONAL:

1.0

OTHER:

0.0

CHECK APPROPRIATE BOX(ES)

- (a) Human subjects (b) Human tissues (c) Neither
- (a1) Minors
- (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Using standard recombinant DNA and yeast transformation techniques, a number of expression vectors have been constructed in order to get the regulated overproduction of sis-related proteins in Saccharomyces cerevisiae. Both retroviral (v-sis) and human (c-sis) cDNA sequences have been used for these constructs. Independently of the type of promoter used, it seems that only extremely low levels of the sis protein accumulate in the yeast cells. Thus, sis proteins are not detectable by radioimmunoprecipitation in labeled yeast cell lysates. Under certain conditions, vectors based on the promoter and processing signals of the yeast mating-type alpha structural gene seem to release sis-related proteins into the culture medium. Therefore, at the present time efforts are concentrated in the detection of the proteins by mitogenic assays on quiescent NIH/3T3 and other PDGF-responsive cells.

The inducible lambda bacteriophage-derived promoter PL is currently being used in pBR322-based vectors to attempt the regulated overproduction of the gp70 protein product of the v-fgr oncogene, a member of the tyrosine-kinase family, in Escherichia coli. For this purpose, either the entire v-fgr coding region or portions of it encompassing its three distinguishable domains (gag, actin and tyrosine kinase-related) have been used to construct the expression systems. Radioimmunoprecipitation and western-blot techniques are being used for detection of the protein species synthesized by the bacterial cells.

PROJECT DESCRIPTIONNames, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

V. Notario	Visiting Associate	LCMB	NCI
K. C. Robbins	Act. Chief, Molecular Genetics Section	LCMB	NCI

Objectives:

To isolate and characterize oncogenes and to develop nonhuman model systems to study the mechanisms of oncogenesis.

Methods Employed:

Standard molecular and biological techniques for nucleic acid isolation, gene enrichment, Southern transfer, molecular hybridization, recombinant DNA techniques, restriction enzyme mapping of cloned genes, DNA sequencing and immunoprecipitation are routinely used. In addition, methods related to yeast genetics, such as yeast transformation, protoplasting, conjugation, tetrad analysis and gene mapping, have been set up for this project.

Major Findings:

With regard to the project related to the sis oncogene, the main accomplishment has been the construction of a wide variety of yeast expression plasmids for overproduction of viral and human sis proteins. These experiments will lead to their purification to homogeneity and their structural and functional characterization. Preliminary results obtained with proteinase-deficient strains suggest that the stability and/or structure of the sis message prevent(s) the accumulation of the proteins in yeast cells.

In addition, preliminary experiments have been carried out with a set of constructs for expression of the v-fgr protein product in bacteria. Overproduction of this protein will lead not only to preparation of specific antibodies which will allow study of the properties of the viral protein per se, but also, and more importantly, to the detection and characterization of the protein product of the normal human cellular counterpart from cells in which it is expressed.

Publications:

Leal, F., Igarashi, H., Gazit, A., Williams, L. T., Notario, V., Tronick, S. R., Robbins, K. C. and Aaronson, S. A.: Mechanism of transformation by an oncogene coding for a normal growth factor. In Proc. UCLA Symposia on Molecular & Cellular Biology. Biochemical and Molecular Epidemiology of Cancer. (In Press)

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Notario, V., Santos, E., Sukumar, S., Martin-Zanca, D. and Barbacid, M.:
El papel de los oncogenes en la carcinogenesis humana. (The role of oncogenes
in human carcinogenesis.) In Genetica Molecular. Madrid, Spain, Instituto de
Ciencias del Hombre, 1985, pp. 217-236.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05306-04 LCMB

PERIOD COVERED

October 1, 1985 to September 30, 1986

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Properties of the ras p21 Proteins and Identification of db1 Oncogene Product

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	S. K. Srivastava	Visiting Associate	LCMB	NCI
Others:	A. Eva	Visiting Scientist	LCMB	NCI
	J. C. Lacal	Visiting Fellow	LCMB	NCI
	S. A. Aaronson	Chief	LCMB	NCI

COOPERATING UNITS (if any)

None

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INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

1.0

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1.0

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0.0

CHECK APPROPRIATE BOX(ES)

- (a) Human subjects (b) Human tissues (c) Neither
 (a1) Minors
 (a2) Interviews

SUMMARY OF WORK (Use standard un-reduced type. Do not exceed the space provided.)

Project 1: In our continued effort to understand the structure-function relationship of ras p21, we have made inframe deletions of 4-5 amino acids in the region of p21 protein, which has been mapped by Lacal and Aaronson (Mol. Cell. Biol. 37, 1002, 1986) as the epitope for anti-ras p21 monoclonal antibody, Y13-259. This antibody can revert the transforming effect of p21 and can also block the initiation of DNA synthesis (Mulcahy et al., Nature 313, 21, 1985). However, Y13-259 does not directly interfere with known biochemical properties of p21. These studies suggest that this antibody may affect some as yet undetected function of p21. To gain insights into the function of Y13-259 binding region of p21, these mutants are currently being analyzed for their biochemical and biological properties.

Project 2: A novel oncogene, db1, isolated from a human diffuse B-cell lymphoma, has been previously shown to confer the transformed phenotype on NIH/3T3 cells. Our present studies were undertaken in an effort to identify the db1 oncogene product and to characterize some of its structural and functional properties. A 66,000 dalton protein (p66) has been identified in cells that have acquired db1 oncogene, while it is not present in cells transformed by other oncogenes or in untransformed NIH/3T3 cells. Furthermore, it is established that p66 is encoded by db1 oncogene. p66 is a phosphoprotein with serine a major site of phosphorylation and it is a cytoplasmic protein as judged by its subcellular distribution.

PROJECT DESCRIPTIONNames, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

S. K. Srivastava	Visiting Associate	LCMB	NCI
A. Eva	Visiting Scientist	LCMB	NCI
J. C. Laca1	Visiting Fellow	LCMB	NCI
S. A. Aaronson	Chief	LCMB	NCI

Objectives:

1. Construction of p21 mutants in the region of protein which forms epitope for monoclonal anti-p21 antibody Y13-259. Characterization of biochemical and biological properties of p21 mutants in eukaryotic and bacterial expression systems.
2. Identification and characterization of the protein encoded by dbl, a novel human oncogene.

Methods Employed:

1. Column chromatographic procedures involved in purification of proteins.
2. Immunoprecipitation of p21 proteins using monoclonal and anti-oligopeptide antibodies and their analysis on SDS-polyacrylamide gel electrophoresis and western blot analysis of p21 proteins.
3. In vitro assays for GTP binding autophosphorylation and GTPase activities of p21.
4. In vitro deletion mutagenesis using synthetic oligonucleotides.
5. Analysis of dbl transfectants for transformation specific antigen using sera from mice bearing tumors induced by dbl oncogene transformed NIH/3T3 cells.
6. General methods for biochemical analysis of oncogene product.

Major Findings:

1. p21 mutants have been obtained in the region of protein which is an epitope for monoclonal antibody Y13-259. Biological and biochemical analyses of the mutants are in progress.
2. Utilizing dbl tumor-bearing mice, we have identified the product of dbl oncogene. Initial characterization of dbl p66 distinguishes it from the products of previously reported oncogene.

Publications:

1. Lical, J. C., Srivastava, S. K., Anderson, P. S. and Aaronson, S. A.: ras proteins with high or low GTPase activity can behave as transforming proteins following microinjection into NIH/3T3 cells. Cell 44: 609-617, 1986.
2. Needleman, S. W., Kraus, M. K., Srivastava, S. K., Levine, P. H. and Aaronson, S. A.: High frequency of N-ras activation in acute myelogenous leukemia. Blood 67: 753-757, 1986.
3. Srivastava, S. K., Lical, J. C., Reynolds, S. H. and Aaronson, S. A.: Antibody of predetermined specificity to a carboxy-terminal region of H-ras gene products inhibits their guanine nucleotide-binding function. Mol. Cell. Biol. 5: 3316-3319, 1985.
4. Tronick, S. R., Eva, A., Srivastava, S., Kraus, M., Yuasa, Y. and Aaronson, S. A.: The role of human ras proto-oncogenes in cancer. In Castellani, A. (Ed.): Epidemiology and Quantitation of Environmental Risk in Humans from Radiation and Other Agents. New York, Plenum Press, 1985. (In Press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
 NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05362-03 LCMB

PERIOD COVERED

October 1, 1985 to September 30, 1986

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Serum-Free Culture of Transformed and Untransformed Mouse Keratinocytes

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	J. Falco	Medical Staff Fellow	LCMB NCI
Others:	S. A. Aaronson	Chief	LCMB NCI
	P. P. Di Fiore	Visiting Fellow	LCMB NCI
	W. G. Taylor	Research Biologist	LCMB NCI

COOPERATING UNITS (if any)

None

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NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

1.0

PROFESSIONAL:

1.0

OTHER:

0.0

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- (a) Human subjects (b) Human tissues (c) Neither
 (a1) Minors
 (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

A serum-free defined media system was developed for the BALB/MK mouse keratinocyte cell line. It was found that this cell line required only two growth factors, insulin and epidermal growth factor (EGF), for growth rates which approached those achievable in serum-containing media supplemented with EGF.

This defined media system was then used to determine the precise growth factor requirements of BALB/MK cells transformed with a variety of oncoviruses. It was found that nearly all of the viral transformants were able to grow in the absence of EGF. However, different oncoviruses varied in how efficiently they abrogated EGF dependence. H-ras-, K-ras-, fgr- and abl-transformed cells became entirely EGF-independent. Fms-, mos- and erbB-transformed cells grew in the absence of EGF, but grew better when EGF was present. Cells transformed with fgr and mos were also able to grow in the absence of EGF.

This defined media system has also been adapted as a method of stringently selecting for cells which escape from growth factor requirements following transformation.

PROJECT DESCRIPTIONNames, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

J. Falco	Medical Staff Fellow	LCMB	NCI
S. A. Aaronson	Chief	LCMB	NCI
P. P. Di Fiore	Visiting Fellow	LCMB	NCI
W. G. Taylor	Research Biologist	LCMB	NCI

Objectives:

To determine how oncogenic transformation affects the growth factor requirements of epithelial cells.

Methods Employed:

Cells: The BALB/MK mouse keratinocyte cell line, developed in this laboratory, was employed for this study.

Preparation of plates; plating of cells: 60 mm tissue culture dishes were precoated with poly-D-lysine, then human fibronectin ($1 \mu\text{g}/\text{cm}^2$). Cells to be plated were grown in serum-containing media, trypsinized, and the trypsin neutralized with soybean trypsin inhibitor prior to plating; 30,000 cells were plated/dish.

Media and supplements: A 50:50 mix of minimal essential media (MEM) and F-12 media, both low calcium, was used. This media was supplemented with insulin (5 ng/ml), transferrin (5 ng/ml), selenium 5 ng/ml, epidermal growth factor (EGF)(5 ng/ml), and ethanolamine (0.2 mM).

Viral transformants: Low passage BALB/MK cell lines chronically infected with oncogenic retroviruses and selected for EGF-independent growth in the presence of serum were tested for growth in complete serum-free media or in this media lacking either insulin, EGF or ethanolamine.

Harvesting and staining of plates: When the fastest growing plates had achieved confluence, all plates were fixed in 70% ethanol and stained with Giemsa stain.

Selection of erbB transformants: BALB/MK cells were infected with a viral construct containing the erbB gene and driven by Moloney LTRs. Infected cells were then selected for EGF-independent growth either in serum or serum-free media. The growth factor requirements of clones thus selected were compared.

Major Findings:

Growth factor requirements of untransformed BALB/MK cells: It was found that BALB/MK cells had absolute requirements for insulin, EGF, and ethanolamine. Growth rates in media containing all of these factors approached those achievable in serum-containing media. In contrast to other cell lines, hydrocortisone and a partially purified commercial preparation of keratinocyte growth factor did not improve the growth of BALB/MK cells. Bovine pituitary extract inhibited their growth.

Effects of substrate: Precoating of the tissue culture dishes with poly-D-lysine improved the plating efficiency and subsequent growth of serum-free BALB/MK cells. Further improvement in growth was achieved when the poly-D-lysine coated plates were then coated with fibronectin prior to plating of cells. This second precoating was subsequently found to be especially important for the attachment and growth of virally transformed cells.

Growth factor requirements of viral transformants.

EGF requirements: BALB/MK cells infected with fes- and sis-containing viruses were unable to grow without EGF. Cells transformed with fms, mos and src were able to grow in the absence of EGF but grew better when EGF was present. Transformation with abl, H-ras, K-ras and fgr resulted in equal growth in the presence or absence of EGF.

Insulin requirements: fgr and mos transformants exhibited significant growth in the absence of insulin. All other viral transformants showed little or no growth in the absence of insulin.

Growth factor requirements of serum-selected vs. serum-free selected erbB transformants. BALB/MK cells transformed with a viral construct containing the erbB gene and selected for EGF-independent growth in the presence of 8% fetal calf serum were able to grow serum-free in the absence of EGF, but grew better when EGF was present. They exhibited little or no growth in the absence of insulin.

By contrast, serum-free erbB transformants selected for EGF-independent growth were able to grow equally well in the presence or absence of EGF. In addition, two out of three of these serum-free selected clones exhibited significant growth in the absence of insulin and in the absence of both growth factors.

Publications:

Di Fiore, P. P., Falco, J., Borrello, I., Weissman, B. and Aaronson, S. A.: Calcium signal for BALB/MK keratinocyte terminal differentiation counteracts EGF very early in the EGF-induced proliferative pathway. Proc. Natl. Acad. Sci. USA (In Press)

Grimaldi, G., Di Fiore, P. P., Kajtanicik-Locatelli, E., Falco, J. and Blasi, F.: Modulation of urokinase plasminogen activator gene expression during the transition from quiescent to proliferative state in normal mouse cell. EMBO J. 5: 855-861, 1986.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05363-03 LCMB

PERIOD COVERED

October 1, 1985 to September 30, 1986

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Analysis of a Proto-oncogene Encoding a Putative Growth Factor Receptor

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	C. R. King	Staff Fellow	LCMB	NCI
Others:	M. H. Kraus	Visiting Fellow	LCMB	NCI
	P. P. Di Fiore	Visiting Fellow	LCMB	NCI
	O. Segatto	Visiting Fellow	LCMB	NCI
	G. Kruh	Medical Staff Fellow	LCMB	NCI

COOPERATING UNITS (if any)

None

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SECTION

Molecular Biology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

1.0

PROFESSIONAL:

1.0

OTHER:

0.0

CHECK APPROPRIATE BOX(ES)

- (a) Human subjects (b) Human tissues (c) Neither
 (a1) Minors
 (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Using cloned v-erbB as a probe, we have detected abnormalities of two closely related proto-oncogenes in human tumor samples. Abnormalities of the c-erbB 1 gene, the epidermal growth factor receptor, have been identified as gene amplification without apparent abnormalities of the mRNA. Gene amplification in a human mammary carcinoma allowed us to identify and characterize a second and distinct member of this proto-oncogene family, c-erbB 2. The latter is currently under intensive study at the level of the gene mRNA and protein to determine its functional role in both normal and malignant cells.

Recently we have used a similar approach to isolate and partially characterize a second member of the abl proto-oncogene family, termed arg, an abl-related gene.

PROJECT DESCRIPTIONNames, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

C. R. King	Staff Fellow	LCMB	NCI
M. H. Kraus	Visiting Fellow	LCMB	NCI
P. P. Di Fiore	Visiting Fellow	LCMB	NCI
O. Segatto	Visiting Fellow	LCMB	NCI
G. Kruh	Medical Staff Fellow	LCMB	NCI

Objectives:

To identify how the c-erbB 2 gene may contribute to the neoplastic process utilizing two strategies. First, we are exploring the structure of this gene and its product in samples derived from human tumors. Second, we are exploring the types of genetic alteration which can activate the gene to cause neoplastic transformation when introduced into NIH/3T3 cells in culture.

The similarity of the v-erbB 2 gene to the EGF receptor suggests its normal function is to encode a receptor for an unknown growth factor. Efforts are underway which are designed to determine the nature of this putative ligand.

To extend our knowledge to human proto-oncogenes, the characterization of arg, or abl-related gene, will be continued at the level of mRNA structure by cDNA cloning.

Methods Employed:

A major effort has been made to develop cDNA clones corresponding to the full length of the c-erbB 2 mRNA. To isolate such clones, probes were generated from the genomic clone pMAC117 and used to screen libraries from normal human fibroblast as well as MCF-7 tumor cell lines. Individual clones have been assembled into a complete cDNA. This cDNA has been used as a hybridization probe of the DNA and RNA of human tumor material and human tumor cell lines in order to detect abnormalities of gene copy number and transcript size. The cDNAs have also been incorporated into cloned retroviral expression vectors which will result in the expression in all or selected portions of the mRNA. These vectors can be introduced into NIH/3T3 cells in culture using CaPO₄ precipitation and the neoplastic transformation of these cells monitored. Manipulation of the cDNA by truncation and site-specific mutagenesis is in progress.

Antibodies directed against synthetic peptides have been generated which can recognize the c-erbB 2 gene product both in immunoprecipitation as well as western blot assays. These antibodies can be used to detect the extent of protein phosphorylation. The extent to which the protein can autophosphorylate can be used as an assay for the presence of the putative ligand.

Major Findings:

1. Abnormalities of the EGF receptor gene have been identified in human tumor cell lines which result in gene amplification but apparently normal mRNA at high levels.

2. Complete cDNA corresponding to the mRNA of the erbB 2 gene has been isolated.
3. Abnormalities of the c-erbB 2 gene have been identified in human tumor samples and human tumor cell lines from a mammary carcinoma. These abnormalities are apparent gene amplification of the c-erbB 2 gene resulting in overexpression without apparent rearrangement.
4. Overexpression without amplification of the v-erbB 2 gene has been detected in tumor cell lines derived from a human mammary carcinoma.
5. Expression of truncated c-erbB 2 cDNAs in NIH/3T3 cells leads to rapid neoplastic transformation.
6. Antibodies capable of detecting the c-erbB 2 protein product in both immunoblot and immunoprecipitation have been developed.
7. Autophosphorylation of c-erbB 2 product has been detected in cells of membrane preparation and forms the basis of an assay for a putative ligand.
8. Arg, an abl-related proto-oncogene, has been identified and partially characterized.

Publications:

King, C. R., Giese, N. A., Kraus, M. H., Robbins, K. C. and Aaronson, S. A.: Oncogenes as growth factors and growth factor receptors: genetic studies of v-sis and a novel erbB-related gene. In Galeotti, T., Cittadini, A., Neri, G., Papa, S. and Smets, L. A. (Eds.): Cell Membranes and Cancer. Amsterdam, Elsevier Science Publishers, 1985, pp. 411-416.

King, C. R., Giese, N., Robbins, K. C. and Aaronson, S. A.: In vitro mutagenesis of the v-sis transforming gene defines functional domains of its PDGF-related product. Proc. Natl. Acad. Sci. USA 82: 5295-5299, 1985.

King, C. R., Kraus, M. H. and Aaronson, S. A.: Amplification of a novel v-erbB-related gene in a human mammary carcinoma. Science 229: 974-976, 1985.

King, C. R., Kraus, M. H., Williams, L. T., Merlino, G. T., Pastan, I. H. and Aaronson, S. A.: Human tumor cell lines with EGF receptor gene amplification in the absence of aberrant sized mRNAs. Nucleic Acids Res. 13: 8477-8486, 1985.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
 NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
 Z01CP05366-03 LCMB

PERIOD COVERED
 October 1, 1985 to September 30, 1986

TITLE OF PROJECT (60 characters or less. Title must fit on one line between the borders.)
 Studies on the Mechanisms of Oncogene Activation in Human Tumors

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)
 P.I.: S. A. Aaronson Chief LCMB NCI

Others: M. H. Kraus Visiting Fellow LCMB NCI
 C. R. King Staff Fellow LCMB NCI

COOPERATING UNITS (if any)
 N. Popescu and S. Amsbaugh, Laboratory of Biology, NCI

LAB/BRANCH
 Laboratory of Cellular and Molecular Biology

SECTION
 Molecular Biology Section

INSTITUTE AND LOCATION
 NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS: 1.0	PROFESSIONAL: 1.0	OTHER: 0.0
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CHECK APPROPRIATE BOX(ES)
 (a) Human subjects (b) Human tissues (c) Neither
 (a1) Minors
 (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Employing relaxed stringency conditions in Southern blot hybridization experiments with v-erbB as a probe, we have previously identified and partially isolated a novel human v-erbB-related gene which was amplified in a primary mammary adenocarcinoma (mac) and was distinct from the EGF receptor gene. Molecular cloning of a 6-kb genomic Eco RI fragment of mac and nucleotide sequence analysis defined two putative exons with closer homology to v-erbB and human EGF receptor than to other reported tyrosine kinases.

Using an exon-specific genomic mac probe, we identified a 4.8-kb specific mRNA in A431 cells distinct from the three major EGF receptor gene transcripts in this cell line. Expression of mac was observed in a broad spectrum of human tissues of both epithelial and mesodermal origin. In vitro autokinase activity was intrinsic to the 185,000 dalton protein product immunoprecipitated by a specific peptide antibody. In order to investigate the normal structure and potential alterations of mac in human mammary neoplasia, we isolated the entire coding region by cDNA cloning. Subsequent Southern and northern blot analysis of human mammary tumors and tumor cell lines using cDNA probes revealed gene amplification of mac with overexpression in 6/62 cases. Moreover, we observed overexpression of apparently normal size mac mRNA without detectable gene amplification in 3/16 mammary tumor cell lines, suggesting a mac overexpression due to transcriptional dysregulation in these cases. In addition, using EGF receptor cDNA probes, 3/50 mammary tumors and tumor cell lines exhibited gene amplification with overexpression of an apparently normal size transcript.

PROJECT DESCRIPTIONNames, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

S. A. Aaronson	Chief	LCMB	NCI
M. H. Kraus	Visiting Fellow	LCMB	NCI
C. R. King	Staff Fellow	LCMB	NCI

Objectives:

1. To analyze human tumor DNAs for structural gene alterations leading to overexpression of proto-oncogenes.
2. To identify unknown members of proto-oncogene families by relaxed stringency hybridization based on partial sequence homology to known proto-oncogenes.
3. To investigate normal molecular structure of gene and gene product of mac, a novel member of the tyrosine kinase gene family, which was identified due to sequence homology with v-erbB and amplification in a human mammary carcinoma.
4. To elucidate gene alterations of mac and the EGF receptor associated with human mammary neoplasia.

Methods Employed:

(1) Southern blotting; (2) northern blotting; (3) molecular cloning, using phage and bacterial systems; cDNA cloning; (4) nucleotide sequence analysis, according to Maxam and Gilbert and by dideoxy chain termination method; (5) immunoprecipitation, western blotting, phosphorylation assay; (6) DNA transfection by calcium phosphate coprecipitation.

Major Findings:

1. Identification and partial isolation of a novel v-erbB-related human gene, mac, distinct from the EGF receptor and amplified in a human mammary carcinoma.
2. A 4.8-kb mac mRNA is expressed in a variety of tissues, including cells of epithelial and mesodermal origin.
3. In vitro autokinase activity is associated with the 185,000 dalton protein product of mac.
4. The entire coding region of mac was isolated by cDNA cloning.
5. Gene amplification with overexpression of an apparently normal size transcript and overexpression of mRNA without gene amplification in human mammary tumors and tumor cell lines indicate a potential involvement of mac in the pathway of human mammary neoplasia.

6. Gene amplification and overexpression of the EGF receptor in human mammary tumor cells suggest that gene alteration leading to the overexpression of the EGF receptor is associated with human mammary neoplasia as well.

Publications:

King, C. R., Giese, N. A., Kraus, M. H., Robbins, K. C. and Aaronson, S. A.: Oncogenes as growth factors and growth factor receptors: genetic studies of v-sis and a novel erbB-related gene. In Galeotti, T., Cittadini, A., Neri, G., Papa, S. and Smets, L. A. (Eds.): Cell Membranes and Cancer. Amsterdam, Elsevier Science Publishers, 1985, pp. 411-416.

King, C. R., Kraus, M. H. and Aaronson, S. A.: Amplification of a novel v-erbB-related gene in a human mammary carcinoma. Science 229: 974-976, 1985.

King, C. R., Kraus, M. H., Williams, L. T., Merlino, G. T., Pastan, I. H. and Aaronson, S. A.: Human tumor cell lines with EGF receptor gene amplification in the absence of aberrant sized mRNAs. Nucleic Acids Res. 13: 8477-8486, 1985.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05456-02 LCMB

PERIOD COVERED

October 1, 1985 to September 30, 1986

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Transformation Induced by Viral and Cellular fgr Oncogenes

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	M. S. C. Cheah	Medical Staff Fellow	LCMB NCI
Others:	K. C. Robbins	Chief, Molecular Genetics Section	LCMB NCI
	S. R. Tronick	Chief, Gene Structure Section	LCMB NCI
	S. Katamine	Visiting Fellow	LCMB NCI

COOPERATING UNITS (if any)

None

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NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

1.0

PROFESSIONAL:

1.0

OTHER:

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- (a1) Minors
- (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The GR-FeSV onc gene, v-fgr, appears to contain genes coding for actin as well as a tyrosine-specific protein kinase. In an effort to understand the role of the actin domain in the transforming ability of the virus, a series of mutants with deletions in their gag and/or actin sequences were constructed and tested for their ability to transform NIH/3T3 cells. Preliminary data suggest that the actin domain has little effect on transforming activity in vitro but that the carboxy terminus of the gag sequences might be important for membrane binding and transformation.

Expression of the human fgr proto-oncogene is limited to Burkitt's lymphomas naturally infected with Epstein-Barr virus (EBV) but not to EBV-negative Burkitt's. Normal umbilical cord or peripheral blood lymphocyte lines established in vitro by EBV infection also contain detectable c-fgr mRNA. A 50-fold increase in steady state mRNA concentration is observed when uninfected Burkitt's lymphoma cell lines are deliberately infected with EBV. These findings demonstrate for the first time the induction of a proto-oncogene in response to infection by a DNA tumor virus.

Efforts to identify normal sources of fgr proto-oncogene expression have revealed that high levels of c-fgr mRNA are detected in monocytes as well as resting polymorphonuclear leukocytes (PMNs). Although high levels of c-fgr mRNA are present in resting PMNs, the fgr proto-oncogene is transcriptionally inactive, implying that it is synthesized at an earlier stage of granulocytic maturation and is found in the mature PMN as a stable mRNA species. These findings suggest an important role for the fgr proto-oncogene in some facet of granulocytic maturation or function.

PROJECT DESCRIPTIONNames, Titles, Lab and Institute Affiliations of Professional Personnel Engaged on this project:

S. C. Cheah	Medical Staff Fellow	LCMB	NCI
K. C. Robbins	Chief, Molecular Genetics Section	LCMB	NCI
S. R. Tronick	Chief, Gene Structure Section	LCMB	NCI
S. Katamine	Visiting Fellow	LCMB	NCI

Objectives:

1. To study the molecular mechanisms by which GR-FeSV transforms cells.
2. To elucidate the role of the human fgr proto-oncogene in tumorigenesis.
3. To determine the normal function of the human fgr proto-oncogene.

Methods Employed:

Standard biochemical techniques for DNA and RNA isolation and analysis, restriction enzyme analysis, agarose and SDS-PAGE gel electrophoresis, molecular hybridization techniques to analyse genes, standard gene cloning techniques, S1 nuclease protection assays, in situ hybridization with RNA probes, radioimmunoassays for quantitative and qualitative characterization of gene products, peptide synthesis and antibody production, standard recombinant techniques for construction of mutant viruses, standard cell culture techniques, DNA transfections onto NIH/3T3 and COS cells, *in vitro* translation and nuclear run-off assays.

Major Findings:

1. To determine the role of the actin domain of v-fgr in the biologic activity of the virus, a series of mutant viruses were constructed with deletions in their gag and/or actin sequences. These mutants were tested for their ability to transform NIH/3T3 cells in a transfection assay. Preliminary data suggest that the actin domain has little effect on the transforming activity *in vitro* but that the carboxy terminus of the gag sequences might be important for membrane association and transformation.
2. Studies to determine the expression of the human fgr proto-oncogene in a variety of tumors have revealed that certain lymphomas but not sarcomas or carcinomas express a 3-kb fgr-related mRNA. This transcript is detected in Burkitt's lymphoma cell lines naturally with Epstein-Barr virus (EBV) but not in EBV-negative Burkitt's lymphoma cells. Normal umbilical cord or peripheral blood lymphocyte lines established *in vitro* by EBV infection also contain detectable c-fgr mRNA. Moreover, a 50-fold increase of the steady state c-fgr mRNA concentration is observed when uninfected Burkitt's lymphoma cell lines are deliberately infected with EBV. These results are consistent with the possibility that one step in EBV-induced B-cell immortalization involves transcriptional activation of the fgr proto-oncogene in response to an EBV-encoded function.

3. Efforts to identify normal sources of fgr proto-oncogene expression have revealed a lack of c-fgr mRNA in a wide variety of tissues. However, high levels of c-fgr mRNA were detected in a component of normal blood mononuclear cells. We have identified monocytes as the component expressing c-fgr mRNA. In addition, high levels of c-fgr mRNA were expressed by peripheral blood resting polymorphonuclear leukocytes (PMNs). Although high levels of c-fgr mRNA were present in resting PMNs, the fgr proto-oncogene was transcriptionally inactive as judged by nuclear run-off analysis. Thus it appears the fgr proto-oncogene mRNA is synthesized at an earlier stage of granulocyte/monocyte maturation and is found in the mature resting PMN as a stable mRNA species. Based upon the limited distribution of the fgr proto-oncogene mRNA in normal tissues and its apparent narrow window of synthesis in granulocyte/monocyte precursors, our findings suggest an important role for the fgr proto-oncogene in some facet of granulocyte/monocyte maturation or differentiated function.

Publications:

1. Cheah, M. S. C., Igarashi, H., Leal, F., Naharro, G. and Robbins, K. C.: Growth factor mediated proliferative pathways and the neoplastic process. Cancer Invest. (In Press)
2. Cheah, M. S. C., Ley, T. J., Tronick, S. R. and Robbins, K. C.: fgr proto-oncogene mRNA induced in B lymphocytes by Epstein-Barr virus infection. Nature 319: 238-240, 1986.
3. Cheah, M. S. C., Ley, T. J., Tronick, S. R. and Robbins, K. C.: Induction of fgr proto-oncogene mRNA in B lymphocytes as a consequence of EBV infection. In Potter, M. (Ed.): Current Topics in Microbiology and Immunology. (In Press)
4. Kawakami, T., Cheah, M. S. C., Leal, F., Igarashi, H., Pennington, C. Y. and Robbins, K. C.: Involvement of polypeptide growth factors and their receptors in the neoplastic process. In Jacobs, J. R. (Ed.): Proceedings of Scientific and Clinical Perspectives in Head and Neck Cancer. (In Press)
5. Tronick, S. R., Popescu, N. C., Cheah, M. S. C., Swan, D. C., Amsbaugh, S. A., DiPaolo, J. A. and Robbins, K. C.: Isolation and chromosomal localization of the human fgr proto-oncogene. Proc. Natl. Acad. Sci. USA 82: 6595-6599, 1985.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01CP05457-02 LCMB

PERIOD COVERED

October 1, 1985 to September 30, 1986

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Oncogenic Activation of EGF Receptor and Related Genes

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	S. A. Aaronson	Chief	LCMB	NCI
Others:	P. P. Di Fiore	Visiting Fellow	LCMB	NCI
	C. R. King	Staff Fellow	LCMB	NCI
	J. H. Pierce	Sr. Staff Fellow	LCMB	NCI
	M. Kraus	Visiting Fellow	LCMB	NCI

COOPERATING UNITS (if any)

None

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SECTION

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INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

1.0

PROFESSIONAL:

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OTHER:

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- (a) Human subjects (b) Human tissues (c) Neither
 (a1) Minors
 (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The mechanisms by which genes normally encoding for growth factor receptors can be "activated" as oncogenes is being studied. A molecular clone of the v-erb gene from the ES4 isolate of avian erythroblastosis virus (AEV) has been constructed into a murine retroviral vector (MuLV) and a virus has been obtained after transfection of this DNA into NIH/3T3 cells and "rescued" with a helper leukemogenic virus. This new murine pseudotype of the v-erb gene is now being used to study how it alters the growth properties and the differentiation program of a number of in vitro systems of murine origin.

Molecular constructions have been engineered into retroviral vectors to test the transforming potential of the recently isolated HER 2 gene (also called neu). The amino truncation of the protein "activates" its transforming ability; a double amino and carboxy truncation does not enhance this latter effect (as suggested in other systems). Virus stocks obtained as described above from this transforming counterpart of the HER 2 gene are now being tested in vivo and in vitro.

PROJECT DESCRIPTIONNames, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

S. A. Aaronson	Chief	LCMB	NCI
P. P. Di Fiore	Visiting Fellow	LCMB	NCI
C. R. King	Staff Fellow	LCMB	NCI
J. H. Pierce	Sr. Staff Fellow	LCMB	NCI
M. Kraus	Visiting Fellow	LCMB	NCI

Objectives:

1. To study the mechanisms of oncogenic activation of genes which normally encode for growth factor receptors. This will lead to a better understanding of how chronically activated mitogenic signals can convert cells from the normal to malignant state; and
2. To generate viruses from the above "activated" gene for in vivo study and in vitro analysis of systems not susceptible to transfection assay, like hematopoietic cells and epithelial cells. These viruses will be used to study how they alter growth properties and the deployment of differentiated functions in differentiated cytotypes.

Methods Employed:

The MuLV genome has been cloned into the PSV₂gpt vector plasmid, thus creating a retroviral vector molecule bearing (in the plasmid) both a bacterial and a eukaryotic selectable marker (ampicillin and gpt, respectively). This molecule has been used as a standard vector for all the constructions to be described.

The v-erb gene from the AEV 11 clone of ES4 has been constructed into the MuLV/gpt molecule using the Pst I-Bam HI site of the vector molecule and cloning a Pst I-RI fragment from AEV 11 together with oligonucleotide-bearing RI-Bam-HI cohesive ends which completed the structure of the v-erb gene. This construction, called m-erb 1 has served as the parental molecule for all of the following constructions.

The m-erb 1 has been cut at an Apo I site before the initiator ATG and the RI after the stop codon. The vector so obtained has been used to clone several other molecules in a way that all of the 5' regulatory regions upstream from the coding regions would be identical in all of the constructions. The available constructions so far include:

- M-erb 1: v-erb into a MuLV retroviral vector
- v-mac 1: N terminal truncated version of the HER 2 gene
- v-mac 2: N and C terminal truncated version of the HER 2 gene
- MuLV/mac: Full length cDNA of the HER 2 gene
- v-erbB/mac 1: Chimeric molecule bearing the tyrosine kinase of v-erb and a 3' region of the HER 2 gene
- v-erb/mac 2: As v-erb/mac 1 but carboxy terminal truncated.

All of these molecules have been transfected into NIH/3T3 and assayed for transforming ability. Upon superinfection with helper viruses (amphotropic strain), stock viruses have been generated for studies in vivo and in vitro.

Major Findings:

The murine erb virus (m-erb 1) transforms cells of different embryonal derivation in mouse. It transforms fibroblasts, cells of hematopoietic derivation (lymphocytes) and induces lymphomas in vivo. This is at variance with what is observed in the chicken, where AEV mainly induces erythroleukemias and sarcomas.

The m-erb 1 virus abrogates the EGF dependence of a system of mouse keratinocytes in vitro and blocks expression of their differentiation pattern.

The N-terminal truncation of the HER 2 gene strongly activates its transforming capability, resulting in transforming activity comparable to that of the most potent known oncogenes (as ras); the double truncation does not enhance this effect, but rather reduces it by about 50%. Chimeric molecules bearing the tk region of erb and the 3' region of HER behaved much as the v-mac 1 or v-mac 2 constructs, thus identifying a region in the 3' region of the HER 2 molecule which is important in conferring specificity.

Publications:

Di Fiore, P. P., Falco, J., Borrello, I., Weissman, B. and Aaronson, S. A.: Calcium signal for BALB/MK keratinocyte terminal differentiation counteracts EGF very early in the EGF-induced proliferative pathway. Proc. Natl. Acad. Sci. (In Press)

Grimaldi, G., Di Fiore, P. P., Kajtanicik-Locatelli, E., Falco, J. and Blasi, F.: Modulation of urokinase plasminogen activator gene expression during the transition from quiescent to proliferative state in normal mouse cell. EMBO J. 5: 855-861, 1986.

Vecchio, G., Di Fiore, P. P., Fusco, A., Colletta, G., Weissman, B. and Aaronson, S. A.: In vitro transformation of epithelial cells by acute retroviruses. In Human Genes and Diseases. Horizons in Biochemistry and Biophysics. Sussex, England, John Wiley & Sons Ltd. (In Press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05458-02 LCMB

PERIOD COVERED

October 1, 1985 to September 30, 1986

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Gene Organization of Equine Infectious Anemia Virus

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	S. A. Aaronson	Chief	LCMB	NCI
Others:	T. Kawakami	Visiting Fellow	LCMB	NCI
	S. R. Tronick	Chief, Gene Structure Section	LCMB	NCI
	J. E. Dahlberg	Research Microbiologist	LCMB	NCI
	M. Wang	Visiting Fellow	LCMB	NCI

COOPERATING UNITS (if any)

A. Yaniv, A. Gazit and L. Sherman, Sackler School of Medicine, Tel Aviv University, Israel

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TOTAL MAN-YEARS:

1.0

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1.0

OTHER:

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- (a) Human subjects (b) Human tissues (c) Neither
 (a1) Minors
 (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The complete nucleotide sequence (8228 bp) of the equine infectious anemia virus (EIAV) was determined by dideoxy chain termination method. Sequencing established the genomic structure of EIAV, namely, gag, pol and env, as major coding sequences. In addition, the presence of the transactivation protein sequences tat and 3'-orf were strongly suggested by comparison with other lentiviral genomes, including AIDS viruses.

PROJECT DESCRIPTIONNames, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

S. A. Aaronson	Chief	LCMB	NCI
T. Kawakami	Visiting Fellow	LCMB	NCI
S. R. Tronick	Chief, Gene Structure Section	LCMB	NCI
J. E. Dahlberg	Research Microbiologist	LCMB	NCI
M. Wang	Visiting Fellow	LCMB	NCI

Objectives:

To study the pathogenic processes of a lentivirus infection. Studies are directed to elucidate the gene structure and function of an equine infectious anemia virus (EIAV). Efforts are also directed to develop vaccines using recombinant DNA technology.

Methods Employed:

General biochemical methods for DNA extraction and purification. Cloning and subcloning of EIAV genome. Dideoxy chain termination sequencing of DNA improved by tyrosine kinase. Expression of EIAV proteins in bacteria.

Major Findings:

1. The complete nucleotide sequence of an EIAV clone was determined (8228 bp).
2. Sequencing showed the expected common features of retroviral genomes, namely, LTR, gag, pol and env.
3. LTR sequence has small stretches of weak homology to other lentivirus LTRs.
4. env coding sequence was reconstructed by sequencing two clones because the clone used for complete sequencing had a frameshift mutation in env.
5. The reconstructed env sequence has stretches of weak homology to other lentivirus env sequences in terms both of amino acid primary sequence and hydrophobic profile.

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
 NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
 Z01CP05459-02 LCMB

PERIOD COVERED
 October 1, 1985 to September 30, 1986

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
 Structural and Functional Characterization of ras p21 Proteins

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)
 PI: S. A. Aaronson Chief LCMB NCI

Others: J. C. Laca! Visiting Fellow LCMB NCI

COOPERATING UNITS (if any)
 Centro de Biologia Molecular, Universidad Autonoma, Cartoblanco, Madrid, Spain;
 Department of Medicine, SUNY, Stony Brook, New York; Laboratory of Cellular
 Carcinogenesis and Tumor Promotion, NCI.

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SECTION
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INSTITUTE AND LOCATION
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TOTAL MAN-YEARS: 1.0	PROFESSIONAL: 1.0	OTHER: 0.0
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 (a1) Minors
 (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

A series of ras p21 proteins carrying activating point mutations at position 12, 59 or 61, as well as the normal protein, have been expressed in E. coli. The purified proteins were analyzed for in vitro and in vivo activities. We have found that there are at least two different potential mechanisms of activation of transforming properties of ras p21 proteins. One is by means of a reduction of the GTPase activity of the protein, as found in some of the activating point mutations at position 12 or 61. Another mechanism involves acceleration of the interchange of bound nucleotides, as found in mutants with substitutions of Ala to Thr at position 59. Both mechanisms are consistent with the hypothesis that ras p21s are G-binding proteins which act through their association with GTP. After hydrolysis of GTP to GDP + Pi, the p21 molecule loses its ability to interact with other component(s) in the pathway which regulates cell growth. In parallel studies, we have expressed in E. coli a series of deletion mutants of the Harvey-ras p21 protein and utilized them to map epitopes recognized by a series of newly generated or previously reported monoclonal antibodies. By means of this strategy, we have been able to map different regions of the ras p21 molecule related to its biochemical activities. Two regions, from 5-69 and 107-164 are related to GTP-binding activity. A region from 70 to 106 was found to be related to the biological activity of the protein. Finally, microinjection experiments have been performed in which the mitogenic activity of ras p21 proteins has been linked to very early pH alterations induced in microinjected cells. Attempts are being made to correlate pH alterations induced by ras p21 proteins and other protein kinase activities.

PROJECT DESCRIPTIONNames, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

S. A. Aaronson	Chief	LCMB	NCI
J. C. Lacal	Visiting Fellow	LCMB	NCI

Objectives:

1. To define domains of the ras p21 molecule involved in its biochemical and biological activities.
2. To precisely define the pathway in which p21 function is required and to understand the mechanism that leads to uncontrolled cell proliferation as a consequence of p21 activation.

Methods Employed:

Expression in *E. coli* of a series of full length, unfused ras p21 proteins with different point mutations at position 12, 59 or 61 and a series of deletion mutants of the Harvey-ras p21 protein. Generation of monoclonal antibodies against the complete p21 protein and mapping of epitopes required by immunoprecipitation of deletion mutants. In vitro enzymatic analysis of purified proteins and in vivo studies by microinjection of NIH/3T3 and Swiss/3T3 cells.

Major Findings:

1. We have defined a novel mechanism of activation of ras p21 proteins. This mechanism consists of increased guanine nucleotide interchange by point mutations which do not alter its GTPase activity.
2. We have localized a new region of the molecule related to its GTP-binding function, located between amino acid residues 153-164 of the molecule.
3. We have established that GTP binding is absolutely required for the biological activity of the protein.
4. We have mapped the epitope recognized by monoclonal antibody Y13-259 in the p21 molecule. This monoclonal has been shown to specifically block the ras p21 biological activity.
5. We have characterized the effect of Y13-259 monoclonal antibody on the p21 protein. Neither the normal nor the transforming ras proteins are affected in their ability to bind GDP or GTP. However, the ability to interchange prebound nucleotides is blocked when monoclonal Y13-259 is complexed to the p21 proteins.
6. We have been able to demonstrate intracellular pH alterations in NIH/3T3 cells after microinjection with transforming p21 proteins. This alteration occurs within five minutes after microinjection, being the earliest effect induced by ras p21 proteins so far detected.

7. We have generated a series of monoclonal antibodies against p21 proteins and localized their epitopes. In vitro activities have been mapped by means of these monoclonals which are localized in two different regions from 5-69 and 107-164 of the p21 sequence.
8. We have described a new system for the characterization of ras p21 molecules by means of circular dichroism of highly purified, bacterially expressed p21 proteins. By means of these studies, we have detected important structural alterations of the p21 protein by point mutations at either 12 or 61.
9. Microinjection of protein kinase C into Swiss/3T3 cells down-regulated by pretreatment with phorbol esters recover their ability to mitogenically respond to phorbol ester treatment. These results demonstrate in vivo that protein kinase C is the actual receptor of phorbol esters and mediates their tumor promoter action.

Publications:

Lacal, J. C. and Aaronson, S. A.: Monoclonal antibody Y13-259 recognizes an epitope of the p21 ras molecule not directly involved in the GTP-binding activity of the protein. Mol. Cell. Biol. 6: 1002-1009, 1986.

Lacal, J. C. and Aaronson, S. A.: Ras p21 deletion mutants and monoclonal antibodies as tools for localization of regions relevant to p21 function. Proc. Natl. Acad. Sci. USA. (In Press)

Lacal, J. C., Anderson, P. S. and Aaronson, S. A.: Deletion mutants of Harvey ras p21 protein reveal the absolute requirement of at least two distant regions for GTP-binding and transforming activities. EMBO J. 5: 679-687, 1986.

Lacal, J. C., Srivastava, S. K., Anderson, P. S. and Aaronson, S. A.: Ras p21 proteins with high or low GTPase activity can efficiently transform NIH/3T3 cells. Cell 44: 609-617, 1986.

Srivastava, S. K., Lacal, J. C., Reynolds, S. H. and Aaronson, S. A.: Antibody of predetermined specificity to a carboxy-terminal region of H-ras gene products inhibits their guanine nucleotide-binding function. Mol. Cell. Biol. 5: 3316-3319, 1985.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01CP05460-02 LCMB

PERIOD COVERED

October 1, 1985 to September 30, 1986

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Molecular Characterization and Regulation of Expression of *c-sis*/PDGF-2 Locus

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: S. A. Aaronson Chief LCMB NCI

Others: C. D. Rao Visiting Fellow LCMB NCI
K. C. Robbins Act. Chief, Mol. Genetics Section LCMB NCI

COOPERATING UNITS (if any)

None

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NCI, NIH, Bethesda, Maryland 20892

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- (a1) Minors
- (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The structure and sequence of the human *c-sis*/PDGF-2 growth factor have been determined by a combination of methods involving cDNA cloning, nuclease S1 mapping and primer extension. Nucleotide sequence analysis revealed that the 3373-nucleotide *c-sis* mRNA contained only a 723-bp coding sequence for the PDGF-2 precursor polypeptide. The coding sequence was flanked by long 5' (1022-bp) and 3' (1625-bp) untranslated sequences. The entire gene is represented by seven exons, the majority of the first exon and entire seventh exon consisting of the noncoding sequences.

The PDGF-2 gene promoter was localized 24 bp upstream of the mRNA start site by nucleotide sequencing and chloramphenicol acetyl transferase (CAT) assays. By using CAT as a marker gene, we have localized negative regulatory sequences upstream as well as downstream of the promoter. Removal of the negative regulatory sequences resulted in the expression of PDGF-2 CAT sequences in fibroblasts which do not express the PDGF-2 transcript. Our studies indicate that strong secondary structures in the 5' noncoding region also regulate expression of the PDGF-2 polypeptide. Thus, we have determined the complete structure of the *c-sis*/PDGF-2 gene. Tissue-specific regulation of expression of this important growth factor is currently being investigated in detail.

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

S. A. Aaronson	Chief	LCMB	NCI
C. D. Rao	Visiting Fellow	LCMB	NCI
K. C. Robbins	Act. Chief, Molecular Genetics Section	LCMB	NCI

Objectives:

Complete characterization of c-sis/PDGF-2 gene transcript, regulatory elements and its tissue-specific regulation of expression.

Methods Employed:

Standard gene cloning and cDNA cloning; S₁ nuclease mapping; primer extension; restriction enzyme analysis; gel electrophoresis; DNA sequencing; library screening; chloramphenicol acetyl transferase assay as a marker; in vitro transcription; and deletion analysis.

Major Findings:

1. The complete structure of the c-sis/PDGF-2 mRNA has been determined by a combination of cDNA cloning, S₁ nuclease mapping and primer extension.
2. c-sis mRNA consists of 3373 nucleotides, of which only 725 bp represented the coding sequence for PDGF-2 precursor polypeptide.
3. The coding sequence is flanked by long 5' (1022-bp) and 3' (1625-bp) noncoding sequences.
4. Enhancer-like TG element is observed downstream of the polyadenylation signal.
5. The transcriptional promoter "TATAAA" of the c-sis gene is localized 24 bp upstream of the cap site by nucleotide sequencing and CAT assay.
6. Negative regulatory sequences that are responsible for the expression of c-sis in nonexpressing cells have been localized both upstream and downstream of the promoter.
7. Detailed studies on the tissue-specific regulation of c-sis/PDGF-2 expression are in progress.
8. Sequences required for polyadenylation of the c-sis mRNA were localized.

Publications:

Rao, C. D., Igarashi, H., Chiu, I.-M., Robbins, K. C. and Aaronson, S. A.: Structure and sequence of the human c-sis/platelet-derived growth factor-2 (sis/PDGF-2) transcriptional unit. Proc. Natl. Acad. Sci. USA 83: 2392-2396, 1986.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05461-02 LCMB

PERIOD COVERED

October 1, 1985 to September 30, 1986

TITLE OF PROJECT (90 characters or less. Title must fit on one line between the borders.)

Characterization of Normal Counterpart of dbl Oncogene

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	A. Eva	Visiting Scientist	LCMB	NCI
Others:	D. Ron	Visiting Fellow	LCMB	NCI
	S. A. Aaronson	Chief	LCMB	NCI
	S. R. Tronick	Chief, Gene Structure Section	LCMB	NCI

COOPERATING UNITS (if any)

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SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The structure and patterns of expression of the normal counterpart of the dbl oncogene were studied. cDNAs corresponding to transcripts of the normal dbl locus were isolated and their nucleotide sequences are now being determined and compared to that of the transforming gene.

Normal and tumor tissue as well as cell lines derived therefrom were screened with dbl cDNA and genomic probes. The transcript was detected in normal human brain, adrenals, testes and ovaries, and its size determined.

Cloning of the whole dbl normal counterpart will allow its comparison with the biologically active gene and its mechanism of activation. It will also allow us to express the protein both in eukaryotic and prokaryotic systems in order to study its normal function.

PROJECT DESCRIPTIONNames, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

A. Eva	Visiting Scientist	LCMB	NCI
D. Ron	Visiting Fellow	LCMB	NCI
S. A. Aaronson	Chief	LCMB	NCI
S. R. Tronick	Chief, Gene Structure Section	LCMB	NCI

Objectives:

To study the mechanism of activation of the human dbl oncogene by comparison between the cDNAs of the biologically active and the normal gene. Efforts will also be made to express the normal cDNA in eukaryotic and prokaryotic systems in order to study its normal function.

Since the pattern of expression of the normal dbl counterpart seems to be rather specific, I will study the cell type in which it is expressed.

Methods Employed:

Methods of RNA extraction from tissues and cell lines were utilized. Northern blotting was used to screen for dbl-related transcripts. cDNA library screening was employed, as well as other molecular cloning techniques, to subclone dbl-related cDNAs. The dideoxy sequencing technique was utilized. Other methods utilized were Southern blotting, restriction enzyme analysis, gel electrophoresis and preparation of high molecular weight DNA.

Major Findings:

Several normal dbl-related cDNA clones were isolated from a human brain cDNA library (a gift from Dr. Lazarini, NINCDS). Together they span 36 kb. (The dbl normal transcript was determined to be 5 kb.) About 3 kb of these sequences have been determined, of which 1.8 kb overlapped the existing cDNA of the transforming gene.

The expression of dbl-related transcripts on human tissues was determined. Of 20 different types of tissues examined, only brain, adrenals, testes and ovaries were positive.

Of the tumor tissue and cell lines examined, only leiomyosarcoma, teratoma and gastrointestinal carcinomas were positive. The level of expression was similar to or less than the levels in the normal tissues.

The normal dbl transcript was detected in human adrenal medulla and brain adrenal medulla, but not in adrenal cortex. These findings, together with the lack of detection of dbl in tumors of central nervous system or neuro-ectodermal origin may indicate a role of dbl in differentiation of cells from neural origin.

Publications: None.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05463-02 LCMB

PERIOD COVERED

October 1, 1985 to September 30, 1986

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Cellular and Oncogene Products which Participate in Growth Regulation

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: W. G. Taylor Research Biologist LCMB NCI
 Others: S. A. Aaronson Chief LCMB NCI

COOPERATING UNITS (if any)

None

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- (a) Human subjects (b) Human tissues (c) Neither
 (a1) Minors
 (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The long-term objective of this program is to understand the mechanisms of cellular changes fundamental to neoplastic transformation. Mammalian cells in culture represent a model with which to study changes in normal growth regulation. Tumor cells have been shown to produce growth factors with mitogenic activity, and gene products of certain tumor viruses are virtually identical to normal cellular homologues which participate in regulation of cell proliferation. Since mammalian cells in culture usually require undefined serum as a mitogenic stimulant, rigorous assessment of the biologic impact of retroviral gene products on growth control first required development of serum-free culture systems. Present studies in serum-free medium with insulin show *sis* gene complements requirements for sustained proliferation. Analysis of other genes linked to transduction of extracellular mitotic stimuli or intracellular sensing mechanisms for DNA, RNA or protein synthesis is in progress.

PROJECT DESCRIPTIONNames, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

W. G. Taylor	Research Biologist	LCMB	NCI
S. A. Aaronson	Chief	LCMB	NCI

Objectives:

Mammalian cells in culture are a biologic model for the study of mechanism(s) by which tumor viruses or specific viral genes subvert normal growth control. Biologic analysis of mitogenic gene products encoded by tumor viruses requires formulation of a culture environment free of undefined growth stimulants such as serum. This developmental objective was largely achieved during the previous fiscal year, though additional changes to enhance sensitivity or compensate for species or tissue type differences can be tested quickly and incorporated easily. The objective of recent studies was to employ this serum-free culture system and determine if specific onc genes qualitatively or quantitatively complement normal growth factor requirements and thereby undermine cellular growth control mechanisms.

Methods Employed:

Methods are adapted as necessary so that growth requirements of NIH/3T3 cells can be compared with NIH/3T3 cells recently transfected with sis, erbB or ras genes. Petri dishes are precoated to augment adhesion and spreading, and commercially available hormones and growth factors are used to supplement a serum-free medium designed in this laboratory for this research objective. Gene complementation is assessed by systematically omitting growth factors, individually or in combinations. Endpoints for these assays are either total cell yield (comparative net increase in cell number) or colony formation. The biologic activity of conditioned medium is tested by mixing experiments. To date, tumorigenicity assays have been done by implantation of graded cell numbers in nude mice.

Major Findings:

At intermediate density (beginning with 1000 cells per cm²) insulin appears critical for continued NIH/3T3 cell proliferation in serum-free medium, together with a second growth factor: EGF, FGF or PDGF. Though EGF or PDGF alone support limited population doublings, we conclude that NIH/3T3 cells require a set (at least two) of growth factors which, without a source of exogenous growth factors (serum), activates an ordered cascade of metabolic events necessary for semiconservative DNA synthesis and mitosis. In contrast, if onc genes transfected into NIH/3T3 cells complement requirements for cell cycle progression, qualitative or quantitative growth differences could be predicted from the putative site of action of the appropriate gene product(s). In fact, both NIH/3T3 and recent onc gene transfectants acutely deprived of serum generally grow more slowly in insulin/growth factor-containing medium, but the proliferative response of the transfectants differs as predicted among genes tested. Nonproducing simian sarcoma virus-transfected NIH/3T3 cells or

NIH/3T3 cells recently transfected with sis gene proliferate rapidly in serum-free medium containing only exogenous insulin. As the sis gene product is PDGF-2 and PDGF is mitogenic, these results are consistent with the conclusion that sis-transfected cells can complement their growth requirements with endogenous synthesis of sufficient PDGF-like material and sustain proliferation without additional exogenous growth factors. Confirmatory studies with even fewer cells to minimize the impact of endogenous products (autocrine or paracrine) yielded the same results with sis transfectants. A quantitatively similar but qualitatively different pattern is seen with NIH/3T3 transfected with erbB and ras genes.

While assessing gene complementation in NIH/3T3 cells transfected with specific onc genes, we noted that several lines grew continuously in serum and factor-free medium and preliminary analyses were done. First, a nonproducing SSV-transfected line secretes a significant amount of mitogenic, PDGF-like activity which is fully neutralized with anti-PDGF antiserum. In contrast, conditioned factor-free growth medium from a recent ras gene transfectant produced <50% of the mitogenic activity, of which only a portion was neutralized by the same antiserum. Second, an orientation study showed conditioned medium from a recent erbB transfectant had significant mitogenic activity for NIH/3T3 cells. These findings strongly suggest simultaneous, perhaps cooperative, expression of a gene which codes for insulin-like activity, or, alternatively, a reduced insulin requirement following transfection. Third, a recent sis transfectant grown in serum and factor-free medium exhibits amplification in tumorigenicity, though whether this corresponds to increased malignant potential or selection of a cell population with a reduced tumor latent period must be determined. Although preliminary, these observations collectively suggest that cell populations bearing a "known" onc gene and grown in a nutritionally adequate medium will synthesize mitogenic activity sufficient for sustained proliferation even after several passages in culture. Whether this is a stable phenotypic alteration or linked to enhanced tumorigenicity remains to be established.

Publications:

None

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01CP05464-02 LCMB

PERIOD COVERED

October 1, 1985 to September 30, 1986

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Characterization and Cloning of a New Human Oncogene

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	G. Vecchio	Expert	LCMB	NCI
Others:	S. A. Aaronson	Chief	LCMB	NCI
	A. Eva	Visiting Scientist	LCMB	NCI
	S. Tronick	Chief, Gene Structure Section	LCMB	NCI

COOPERATING UNITS (if any)

None

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- (a1) Minors
- (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Subgenomic clones previously obtained from a cosmid clone of a new human oncogene, designated dbl for diffuse B cell lymphoma, have been utilized in order to detect the expression of this newly isolated oncogene in fresh human tumors and tumor cell lines. For this screening, a cDNA clone (representing an incomplete copy of the dbl message) obtained from a cDNA library constructed from NIH/3T3 cells in RNAs transfected with the dbl clone, has also been used. Among about 100 RNA preparations screened, positive hybridization signals have been detected by northern blotting with 10 different preparations of either fresh tumors or cell lines. The positive samples included cell lines of clear mesodermal origin, such as fibrosarcomas, rhabdo- and leiomyosarcomas. Cell lines and fresh tumor samples derived from Ewing's sarcoma (a type of tumor whose cell of origin is still controversial) were consistently found to be positive. Further characterization of the oncogene has included the screening of the cDNA library obtained from transfected NIH/3T3 cells in order to isolate the 5' missing portions of the dbl gene. Fragments of cDNA coming from this region of the gene have been obtained and will eventually lead to the construction of a full length cDNA clone.

PROJECT DESCRIPTIONNames, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

G. Vecchio	Expert	LCMB	NCI
S. A. Aaronson	Chief	LCMB	NCI
A. Eva	Visiting Scientist	LCMB	NCI
S. R. Tronick	Chief, Gene Structure Section	LCMB	NCI

Objectives:

1. Identification of the mRNA for the dbl oncogene in human fresh tumors and tumor cell lines;
2. Isolation of full length cDNA clones for the dbl oncogene from cDNA libraries of NIH/3T3 cells transfected with the oncogene;
3. Use constructs containing full length cDNA clone to assess the oncogenic potential and the mechanism of transformation by the dbl oncogene.

Methods Employed:

Methods used include library screening, restriction enzyme analysis, RNA and DNA extraction and purification and gel electrophoresis analysis.

Major Findings:

1. Identification of a class of tumors and tumor cell lines expressing dbl gene transcripts; and
2. Isolation of new cDNA clones containing the 5' region of the dbl oncogene.

Publications:

Vecchio, G., Di Fiore, P. P., Fusco, A., Colletta, G., Weissman, B. and Aaronson, S. A.: In vitro transformation of epithelial cells by acute retroviruses. In Human Genes and Diseases. Horizons in Biochemistry and Biophysics. Sussex, England, John Wiley & Sons Ltd. (In Press)

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01CP05466-01 LCMB

PERIOD COVERED

October 1, 1985 to September 30, 1986

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

The Role of Human Transforming Growth Factor Alpha in Neoplasia

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	E. Finzi	Medical Staff Fellow	LCMB	NCI
Others:	S. A. Aaronson	Chief	LCMB	NCI
	T. Fleming	Guest Researcher	LCMB	NCI
	O. Segatto	Visiting Fellow	LCMB	NCI
	J. Pierce	Sr. Staff Fellow	LCMB	NCI

COOPERATING UNITS (if any)

T. S. Bringman and R. K. Derynck, Department of Molecular Biology, Genentech, Inc., South San Francisco, CA

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- (a1) Minors
- (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

To understand the role of TGF α in transformation, the human TGF α gene was placed under the control of a retroviral LTR and the mouse metallothionein promoter. Transfection of recombinant plasmids onto NIH/3T3 cells did not result in the formation of foci. However, transfected cells were shown to secrete large amounts of TGF α into the medium, to grow to high saturation density, and to have down-regulated receptors to EGF. DNA synthesis by TGF α cell lines was inhibited by anti-human TGF α monoclonal antibodies, suggesting potential ways to inhibit tumor growth in vivo. Comparison of TGF α sublines with cells transfected by a recombinant v-erbB plasmid showed v-erbB cell lines grow better in agar and produced tumors in nude mice faster than did TGF α cell lines.

A recombinant murine retroviral vector containing the human TGF α gene was constructed in order to further study the effect of TGF α in vitro and in vivo. A retroviral construct containing the TGF α gene and a selectable marker, the bacterial Tn5 gene, was transfected into ψ -2 cells. NIH/3T3 cells infected by high titer recombinant virus produced by ψ -2 cells were found to secrete TGF α into the medium. The effect of TGF α viral infection in BALB/MK cells and in nude mice is currently being investigated.

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

E. Finzi	Medical Staff Fellow	LCMB	NCI
S. A. Aaronson	Chief	LCMB	NCI
T. Fleming	Guest Researcher	LCMB	NCI
O. Segatto	Visiting Fellow	LCMB	NCI
J. Pierce	Sr. Staff Fellow	LCMB	NCI

Objectives:

To determine the relevance of TGF α , which is secreted by many human tumor cells, in the pathogenesis of human neoplasia. Studies are directed towards expression of the TGF α gene in different cell types and characterization of the biologic effects of this expression. High titer stocks of ecotropic and amphotropic pseudotyped TGF α recombinant retroviruses are being generated in order to study the effect of TGF α viral infection in nude mice.

Methods Employed:

The TGF α cDNA has been cloned into several eukaryotic expression vectors. DNA-mediated gene transfer (DNA transfection) has been used to introduce cloned DNA into NIH/3T3 cells. Cotransfection of recombinant TGF α plasmids with plasmids containing selectable markers has been used to generate cell lines expressing TGF α . A number of biochemical techniques, including EGF-receptor competition assays, Scatchard analysis, mitogenesis and growth in soft agar are used to characterize cells infected by the TGF α virus.

Major Findings:

Transfection of recombinant TGF α plasmids onto NIH/3T3 cells did not result in the formation of foci. However, cell lines generated by transfection were shown to secrete up to 20 ng/ml of TGF α in the medium. Growth of TGF α -expressing cell lines in serum-free medium was strictly density-dependent, suggesting that TGF α stimulates cell growth by interacting with external receptors. DNA synthesis by TGF α cells was not stimulated by EGF but was inhibited by the addition of anti-human TGF α monoclonal antibody. Scatchard analysis of I 125 I EGF binding showed that TGF α cells were down-regulated for EGF receptors. TGF α sublines grew slightly better than control cell lines in soft agar. Several of the TGF α sublines, but none of the control lines, formed tumors in nude mice in 5 to 7 weeks. By contrast, v-erbB sublines grew better than TGF α sublines in soft agar and consistently formed tumors within 3 to 4 weeks.

A recombinant TGF α retrovirus with a titer of 2×10^6 /ml was generated by transfection of a retroviral construct onto ψ^2 cells. NIH/3T3 cells infected by this virus secrete large amounts of TGF α into the medium. The EGF-dependent epithelial cell line, BALB/MK, which was developed in this laboratory, has been infected with the TGF α virus and shown to grow in the absence of EGF. The mechanism of this factor-independent growth is being investigated. Infection of nude mice with the TGF α virus is currently underway.

Publications: None

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01CP05467-01 LCMB

PERIOD COVERED

October 1, 1985 to September 30, 1986

TITLE OF PROJECT (50 characters or less. Title must fit on one line between the borders.)

Cloning of Human c-fgr Proto-oncogene cDNA

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	K. C. Robbins	Act. Chief, Mol. Genetics Section	LCMB	NCI
Others:	S. Katamine	Visiting Fellow	LCMB	NCI
	S. R. Tronick	Chief, Gene Structure Section	LCMB	NCI
	M. S. C. Cheah	Medical Staff Fellow	LCMB	NCI
	C. D. Rao	Visiting Fellow	LCMB	NCI
	T. Miki	Guest Researcher	LCMB	NCI
	T. Kawakami	Visiting Fellow	LCMB	NCI

COOPERATING UNITS (if any)

None

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 (a1) Minors
 (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

In order to elucidate the structure and function of the human cellular fgr gene, we have chosen to clone cDNA molecules representing the c-fgr transcript. Thus, cDNA was synthesized from peripheral blood mononuclear cell RNA and cloned into the Okayama-Berg expression vector. Utilizing v-fgr DNA fragments as probes, we identified seven cDNA clones. All of these clones were shown to represent human fgr gene transcripts by Southern analysis of genomic DNA as well as northern analysis of peripheral blood mononuclear cell RNA. The DNA sequence of one cDNA clone, 2.3 kb in length, showed 95% homology at the nucleotide level with that of v-fgr.

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

K. C. Robbins	Act. Chief, Mol. Genetics Section	LCMB	NCI
S. Katamine	Visiting Fellow	LCMB	NCI
S. R. Tronick	Chief, Gene Structure Section	LCMB	NCI
M. S. C. Cheah	Medical Staff Fellow	LCMB	NCI
C. D. Rao	Visiting Fellow	LCMB	NCI
T. Miki	Guest Researcher	LCMB	NCI
T. Kawakami	Visiting Fellow	LCMB	NCI

Objectives:

To assess structure and function of the human cellular counterpart of the fgr oncogene, which is in the src gene family, with tyrosine kinase activity. Initial studies are directed toward the cloning of human c-fgr cDNA.

Methods Employed:

Poly(a)-positive RNA was extracted from human peripheral blood white cells. After the high content of c-fgr mRNA in the RNA was confirmed by northern analysis, a cDNA library was constructed by the Okayama-Berg method and then screened by colony hybridization with ³²P-labeled v-fgr DNA fragment as a probe under stringent conditions. Sequencing of cloned DNA was performed with cloning in the M13 vector and the dideoxy sequencing method.

Major Findings:

We obtained seven cDNA clones from 2.5×10^5 colonies of cDNA library screened. They had 1.9, 2.1, 2.1, 2.2., 2.3, 3.8 and 5.3 kb inserts, respectively. The sequence of one clone with the 2.3-kb insert showed 95% homology with the v-fgr sequence.

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01CP05468-01 LCMB
PERIOD COVERED October 1, 1985 to September 30, 1986		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Implications of New Human Tyrosine Kinase Gene, <u>c-slk</u> , on Tumorigenesis		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
PI:	K. C. Robbins	Act. Chief, Mol. Genet. Section LCMB NCI
Others:	T. Kawakami	Visiting Fellow LCMB NCI
	S. A. Aaronson	Chief LCMB NCI
	S. R. Tronick	Chief, Gene Structure Section LCMB NCI
	N. C. Popescu	Microbiologist LB NCI
	S. C. Amsbaugh	Microbiologist LB NCI
COOPERATING UNITS (if any)		
LAB/BRANCH Laboratory of Cellular and Molecular Biology		
SECTION Molecular Biology Section		
INSTITUTE AND LOCATION NCI, NIH, Bethesda, Maryland 20892		
TOTAL MAN-YEARS: 1.0	PROFESSIONAL: 1.0	OTHER: 0.0
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input checked="" type="checkbox"/> (b) Human tissues <input type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) A new member of the human <u>src</u> gene family was isolated on the basis of cross hybridization with the probe <u>v-fgr</u> . Cloning and sequencing the cDNAs showed that the new gene, termed <u>c-slk</u> , encodes 537 amino acid proteins which have extensive homology with other <u>src</u> tyrosine kinase genes but an essentially nonhomologous N-terminal segment of 82 amino acids. The chimeric construct, which has the Gardner-Rasheed feline sarcoma virus (GR-FeSV) sequence at 5' half and <u>c-slk</u> tyrosine kinase domain at 3' half, transformed the mouse fibroblast NIH/3T3. The transformed cells had the chimeric phosphoprotein, p71 <u>gag/slk</u> which has tyrosine kinase in vitro. For the purpose of pursuing the possible involvement of <u>c-slk</u> in human tumorigenesis, the <u>c-slk</u> gene was localized on the long arm of chromosome 6. We are now screening various human tumor DNAs in terms of rearrangement and amplification of <u>c-slk</u> .		

PROJECT DESCRIPTIONNames, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

K. C. Robbins	Acting Chief, Molecular Genetics Section	LCMB	NCI
T. Kawakami	Visiting Fellow	LCMB	NCI
S. A. Aaronson	Chief	LCMB	NCI
S. R. Tronick	Chief, Gene Structure Section	LCMB	NCI
N. C. Popescu	Microbiologist	LB	NCI
S. C. Amsbaugh	Microbiologist	LB	NCI

Objectives:

To study the functional domain structure of human c-slk gene and its normal function. Efforts are also directed to study its potential functions in human malignancies.

Methods Employed:

General biochemical methods for nucleic acid extraction and purification; in vitro mutagenesis techniques using recombinant DNAs; protein expression in bacteria and raising antibody; immunoprecipitation; DNA or RNA blotting and molecular hybridization; DNA cloning (genomic and cDNA); S1 mapping.

Major Findings:

1. A new member of the human src gene family was isolated.
2. The new gene, termed c-slk, can encode 537 amino acid proteins, namely, LTR, gag, pol and env.
3. The tyrosine kinase domain (3' half) of c-slk is highly homologous to other members of the src family. But the 5' terminal portion of 82 residues is essentially nonhomologous.
4. The construct, of which the 5' half is the Gardner-Rasheed feline sarcoma virus (GR-FeSV) gene and the 3' half is the tyrosine kinase domain of c-slk, transformed mouse fibroblast NIH/3T3 cells.
5. Transformed NIH/3T3 cells contained the chimeric phosphoprotein p71gag/slk.
6. p71gag/slk showed tyrosine kinase activity in vitro.

Publications:

Kawakami, T., Cheah, M. S. C., Leal, F., Igarashi, H., Pennington, C. Y. and Robbins, K. C.: Involvement of polypeptide growth factors and their receptors in the neoplastic process. In Jacobs, J. R., Valeriote, F., Crissman, J. and Al-Sarraf, M. (Eds.): Head and Neck Cancer: Scientific Principles and Management. Elsevier. (In Press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01CP05469-01 LCMB
PERIOD COVERED October 1, 1985 to September 30, 1986		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Identification of New Tyrosine Kinase Oncogenes		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
PI:	G. Krueh	Medical Staff Fellow LCMB NCI
Others:	S. A. Aaronson	Chief LCMB NCI
	C. R. King	Staff Fellow LCMB NCI
	M. Kraus	Visiting Fellow LCMB NCI
COOPERATING UNITS (if any) None		
LAB/BRANCH Laboratory of Cellular and Molecular Biology		
SECTION Office of the Chief		
INSTITUTE AND LOCATION NCI, NIH, Bethesda, Maryland 20892		
TOTAL MAN-YEARS: 1.0	PROFESSIONAL: 1.0	OTHER: 0.0
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input checked="" type="checkbox"/> (b) Human tissues <input type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard un-reduced type. Do not exceed the space provided.) <p>In order to understand the role of growth factor receptors in neoplasia, the identification of new oncogenes was attempted. A new gene with extensive homology to v-abl, termed <u>arg</u> (Abelson-related gene), was identified in normal human DNA. This new gene was found to be expressed in several human tissues, as well as a variety of tumor cell lines. Thus, based upon nucleotide sequence diversity and identification of a distinct RNA transcript, <u>arg</u> represents a new functional human gene of the tyrosine kinase family. Investigation of the complete coding sequence of <u>arg</u> should provide insight into its possible function.</p>		

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

G. Kruh	Medical Staff Fellow	LCMB	NCI
S. A. Aaronson	Chief	LCMB	NCI
C. R. King	Staff Fellow	LCMB	NCI
M. Kraus	Visiting Fellow	LCMB	NCI

Objectives:

To study the role of growth factor receptors in the development and pathogenicity of human tumors. This investigation is directed toward identifying new cellular oncogenes which may play a role in neoplasia.

Methods Employed:

Molecular hybridization is performed with DNA extracted from normal human tissue and a probe for a tyrosine kinase-encoding viral oncogene, using the techniques of restriction enzyme analysis and Southern blotting. Gene fragments identified in this fashion are further characterized using the techniques of molecular cloning in bacteriophage, subcloning in bacterial plasmids and nucleotide sequence analysis by the dideoxy chain termination method. Expression of new oncogenes is studied with the techniques of RNA extraction and northern blot analysis.

Major Findings:

A 12.5-kb gene fragment with sequence homologous to v-abl was identified in normal DNA. Analysis revealed that the fragment contains two exons of a gene extensively homologous to, but distinct from, c-abl. This gene was found to be expressed in several tissues as well as a variety of human tumor cell lines. This Abelson-related gene, which we have termed arg, thus represents a new functional human gene.

Publications:

None

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01CP05470-01 LCMB

PERIOD COVERED

October 1, 1985 to September 30, 1986

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Purification and Characterization of Epithelial Cell Growth Factor

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: S. A. Aaronson Chief LCMB NCI

Others: H. Osada Visiting Fellow LCMB NCI

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Cellular and Molecular Biology

SECTION

Molecular Biology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

1.0

PROFESSIONAL:

1.0

OTHER:

0.0

CHECK APPROPRIATE BOX(ES)

- (a) Human subjects (b) Human tissues (c) Neither
- (a1) Minors
- (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Some fibroblasts are known to secrete the growth factor for epithelial cells. To elucidate the properties and the biological role of the growth factor, its proteins were purified. One human fibroblast, M426, produces two proteins (MW 68,000 and MW 18,000), each of which was purified to a homogeneous state by column chromatography and fast protein liquid chromatography (FPLC).

Antibodies of both proteins are currently being made and their amino acid sequences being determined.

PROJECT DESCRIPTIONNames, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

S. A. Aaronson	Chief	LCMB	NCI
H. Osada	Visiting Fellow	LCMB	NCI

Objectives:

The aim of this project is to elucidate the growth control of epithelial cells which might be concerned with carcinoma. It is known that some fibroblasts secrete the growth factor for epithelial cells. To reveal the properties and the biological role of the growth factor and its distribution in human tissues, attempts are being made to purify the components of the factor.

Methods Employed:

The activity of the growth factor is assayed by mitogen assay, which measures the incorporation of ³H-thymidine into quiescent cells after stimulation by the factor.

The proteins are being purified by conventional column chromatography and fast protein liquid chromatography (FPLC). Molecular weight of the proteins was determined by SDS-polyacrylamide gel electrophoresis.

Major Findings:

About 100 conditioned media of human fibroblasts were assayed by mitogen assay. The epithelial cell growth factor was produced in 100% of embryo fibroblasts, 63% of colon fibroblasts, 60% of lung fibroblasts, and 43% of breast fibroblasts, whereas none was produced in skin, glia or thymus fibroblasts.

Two distinct proteins were purified from human embryo fibroblasts. These proteins (MW 68,000 and MW 18,000) were found to stimulate DNA synthesis preferentially in epithelial cells (BALB/M/C) rather than in fibroblasts (NIH/3T3).

Publications:

None

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01CP05471-01 LCMB

PERIOD COVERED

October 1, 1985 to September 30, 1986

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Studies on Oncogenes in Human Gastrointestinal Tumors

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: J. S. Rhim Research Microbiologist LCMB NCI

Others: J. B. Park Visiting Fellow LCMB NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Cellular and Molecular Biology

SECTION

Office of the Chief

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

1.0

PROFESSIONAL:

1.0

OTHER:

0.0

CHECK APPROPRIATE BOX(ES)

- (a) Human subjects (b) Human tissues (c) Neither
- (a1) Minors
- (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

In order to assess the significance of activated oncogenes in the pathogenesis of human gastrointestinal malignancies, especially gastric and hepatic carcinomas, fresh tumors of the stomach, rectum and liver were screened utilizing both DNA transfection and gene amplification techniques. In one of 35 stomach cancers, a threefold amplification of the K-ras gene was detected, and of five rectal cancers, one was positive in the DNA transfection assay. The oncogene detected in the latter was N-ras, with a 61st codon mutation.

PROJECT DESCRIPTIONNames, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

J. S. Rhim	Research Microbiologist	LCMB	NCI
J. B. Park	Visiting Fellow	LCMB	NCI

Objectives:

To study the relevance of cellular oncogenes in pathogenesis and clinical findings of human gastrointestinal tract tumors, with special emphasis on stomach and liver tumors, the most common cancers in Korea. Studies are directed toward identification of the oncogenes involved and the specific molecular events leading to their activation. Specific methods to detect activated oncogenes are being developed and mechanisms to prevent or reverse transformation are being investigated.

Methods Employed:

This laboratory has developed and utilized DNA-mediated gene transfer (DNA transfection) using NIH/3T3 cells as recipients to detect activated cellular transforming genes in malignant tumors. DNA is extracted from human gastrointestinal tumors and used for assay. Morphology is followed by light microscopy after DNA transfection and transformed foci are picked up and propagated for further analysis. A number of biochemical and molecular biological techniques, including immunoprecipitation and restriction endonuclease analysis, are used to characterize the activated oncogene.

Major Findings:

Twenty-eight samples of human gastric tumors were analyzed by DNA transfection. So far, no positives were detected in the DNA transfection assay. However, the N-ras gene was found to be amplified in one of 35 stomach cancers.

Of five human rectal cancers, one was positive in the DNA transfection assay. The isolate had activated N-ras with a 61st codon mutation.

Publications:

None

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01CP05472-01 LCMB

PERIOD COVERED

October 1, 1985 to September 30, 1986

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Role of Cellular and Viral Oncogenes on Cell Growth

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	S. A. Aaronson	Chief	LCMB	NCI
Others:	O. Segatto	Visiting Fellow	LCMB	NCI
	E. Finzi	Medical Staff Fellow	LCMB	NCI
	W. G. Taylor	Research Biologist	LCMB	NCI
	P. P. Di Fiore	Visiting Fellow	LCMB	NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Cellular and Molecular Biology

SECTION

Molecular Biology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

1.0

PROFESSIONAL:

1.0

OTHER:

0.0

CHECK APPROPRIATE BOX(ES)

- (a) Human subjects (b) Human tissues (c) Neither
- (a1) Minors
- (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

In order to assess the role of cellular and viral oncogenes in the mechanisms of control of cell growth, we developed serum-free culture conditions for NIH/3T3 cells, a murine contact-inhibited fibroblast cell line, which has been shown to be susceptible to DNA transfection and is therefore suitable for gene transfer experiments. Epidermal growth factor (EGF) was found to be the major requirement for NIH/3T3 growth in our defined media. We therefore tried to assess whether expression of viral oncogenes or deregulation of normal cellular gene expression in NIH/3T3 cells could alter their EGF requirement. TGF α , v-fos and v-erbB expression were found to release NIH/3T3 cells from EGF dependence, whereas overexpression of the nuclear oncogene pp53 did not alter their growth factor requirements. Expression of TGF α was found to affect EGF requirement in a cell-density dependent fashion, whereas v-fos-transformed NIH/3T3 could grow without EGF at clonal dilutions. Similar experiments are in progress with v-erbB transformed NIH/3T3 cells.

PROJECT DESCRIPTIONNames, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

S. A. Aaronson	Chief	LCMB	NCI
O. Segatto	Visiting Fellow	LCMB	NCI
E. Finzi	Medical Staff Fellow	LCMB	NCI
W. G. Taylor	Research Biologist	LCMB	NCI
P. P. Di Fiore	Visiting Fellow	LCMB	NCI

Objectives:

To study the role of cellular and viral oncogenes on the control of cell growth. Studies are directed toward assessing whether altered expression of genes encoding for proteins with different functions in the growth factor signaling pathway can release cells from the need of exogenously added growth factors in serum-free culture conditions.

Methods Employed:

Culture conditions have been developed which allow clonal growth of NIH/3T3 cells in a chemically defined, serum-free medium containing highly purified growth factors. These culture conditions have been used to study the growth properties of clonal NIH/3T3 cell lines derived by transfection of molecular clones of the *v-erbB* and *v-fos* genomes and of the mouse *pp53* and human $TGF\alpha$ genes inserted in eukaryotic expression vectors. The ability of these transfectants and of control NIH/3T3 clones to grow in selective serum-free media deprived of one or more growth factors is being studied using growth curve and colony formation assays.

Major Findings:

Expression of *v-fos* and *v-erbB* in NIH/3T3 results in morphological transformation in a conventional focus assay. These transformants can grow in serum-free media depleted of EGF. The *v-fos* transformants require only insulin for growth at clonal dilutions. These same conditions do not allow any sustained proliferation of NIH/3T3 control clones. Expression of $TGF\alpha$ does not morphologically transform NIH/3T3 cells but is able to release them from EGF requirements in a cell-density dependent fashion. However, if the $TGF\alpha$ gene is driven by an inducible promoter, cell growth is allowed also at clonal dilutions in EGF-free media, provided the inducing factor is continuously present in the culture media. The *pp53* transfectants do not grow significantly better than control clones in selective serum-free media.

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
 NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05473-01 LCMB

PERIOD COVERED

October 1, 1985 to September 30, 1986

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Studies of Mechanisms of Pathogenesis of Animal Lentiviruses

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	S. R. Tronick	Chief, Gene Structure Section	LCMB	NCI
Others:	M. C. Wang	Visiting Fellow	LCMB	NCI
	S. A. Aaronson	Chief	LCMB	NCI
	J. E. Dahlberg	Research Microbiologist	LCMB	NCI
	T. Kawakami	Visiting Fellow	LCMB	NCI

COOPERATING UNITS (if any)

None

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Laboratory of Cellular and Molecular Biology

SECTION

Molecular Biology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

1.0

PROFESSIONAL:

1.0

OTHER:

0.0

CHECK APPROPRIATE BOX(ES)

- (a) Human subjects (b) Human tissues (c) Neither
 (a1) Minors
 (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

To investigate the mechanism of pathogenesis of animal lentiviruses, a number of subclones of the equine infectious anemia virus (EIAV) genome were subcloned in plasmid vectors. These were used to express EIAV proteins for use in development of more sensitive assays for the detection of EIAV antigens and antisera to the virus.

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

S. R. Tronick	Chief, Gene Structure Section	LCMB	NCI
M. C. Wang	Visiting Fellow	LCMB	NCI
S. A. Aaronson	Chief	LCMB	NCI
J. E. Dahlberg	Research Microbiologist	LCMB	NCI
T. Kawakami	Visiting Fellow	LCMB	NCI

Objectives:

To investigate the molecular mechanisms of lentivirus-induced disease and to develop sensitive assays for detection of lentivirus proteins.

Methods Employed:

Recombinant DNA, standard biochemical analyses of DNA, western blotting and immunoprecipitation.

Major Findings:

Subclones of the EIAV genome were constructed such that sequences representing viral gag, pol, and env genes could be expressed in bacteria under the control of the lac promoter. Using these inducible systems, we detected expression of the EIAV protein in bacteria using acrylamide gel electrophoresis and western blotting. Induction and growth of bacteria are now being optimized to increase protein expression. At the same time, antisera (polyclonal and monoclonal) are being raised against EIAV proteins expressed in these systems.

Publications:

None

ANNUAL REPORT OF

THE LABORATORY OF MOLECULAR ONCOLOGY
BIOLOGICAL CARCINOGENESIS PROGRAM
DIVISION OF CANCER ETIOLOGY
NATIONAL CANCER INSTITUTE

October 1, 1985 through September 30, 1986

The Laboratory of Molecular Oncology plans and conducts research defining the molecular and genetic elements responsible for the development and expression of the malignant phenotype in humans and animals. Towards this end, the Laboratory of Molecular Oncology (1) applies the skills of molecular biology, recombinant DNA technology and the methods of immunology to identify and isolate cellular transforming onc genes from malignant and normal cells and tissues, as well as to characterize the product(s) encoded by these genes; (2) identifies, isolates, characterizes and determines the function(s) of these onc genes as determined by the expression and functional analysis of their specified oncoproteins; (3) develops the molecular probes and methodologies required to identify the target(s) of the onc gene product(s), relative to the process of malignant transformation and the normal mode(s) of cellular action; (4) determines and evaluates the organizational structures and function(s) of the normal cellular homologs (proto-onc genes) of the acute transforming viral onc genes by expression in normal eukaryotic and prokaryotic cellular systems; and (5) examines the molecular controlling elements and mechanisms regulating prokaryotic and eukaryotic gene expression, aiming to adapt, modify and apply this understanding to the expression and control of the neoplastic processes. The Laboratory of Molecular Oncology conducts research on the molecular elements responsible for the development and expression of malignant phenotypes in humans and animals. The Laboratory applies skills in molecular biology, recombinant DNA technology and hybridoma-monoclonal antibody production in a comprehensive program to identify and isolate cellular transforming genes and to characterize products expressed by these genes. This mission is accomplished by bringing together expertise in the diverse disciplines of eukaryotic and prokaryotic virology, molecular biology and genetics. The Carcinogenesis Regulation Section studies the relationship between oncogenic gene expression and the conversion of cells from the normal to the transformed, malignant state. Specific regions of molecularly cloned, acute transforming retrovirus genomes are tested for transforming activity and to determine the molecular mechanisms by which their oncogene products act in concert with cellular factors to activate the neoplastic process. This will be accomplished by examining the organization and products of normal essential cellular proto-onc gene(s) and the mechanism(s) by which these genes are subverted into transforming genes, as well as by comparing them to the organization and expression of their corresponding malignant counterparts. The Cellular Transformation Section investigates the malignant transformation of cells by avian sarcoma viruses, including the function of the virus-coded protein(s) directly responsible for transformation, the primary physiological effects of the functioning protein(s), the sequence of metabolic changes resulting in the altered metabolic profile characteristic of malignant cells, and the metabolic changes necessary for the maintenance of the malignant state. The Microbiology Section investigates the mechanism of cell transformation

using biological, biochemical and immunological techniques. The transforming potential in mammalian cells of specific viral and cellular DNA sequences amplified by cloning in appropriate prokaryotic vectors is determined under selective conditions using characterized markers. Transformation studies are augmented using monoclonal antibodies prepared against various DNA sequences and amplified by cloning in appropriate prokaryotic vectors, and then determined under selective conditions using characterized markers. Transformation studies are also augmented using monoclonal antibodies prepared against various viral- and cell-coded proteins. Specifically, this section develops systems to assay and analyze the mechanism of malignant transformation in human primary and established lines of cells using sequences derived from viral and genomic cellular DNA that may have oncogenic potential. The Molecular Control and Genetics Section conducts studies to understand how gene expression is controlled in the prokaryote, *E. coli*, and its phage, lambda. Their focus is on the elucidation of gene regulation at the levels of transcription initiation, transcription termination, RNA translation and RNA processing. In addition, this section, applying its expertise developed with prokaryotic systems, studies the basic molecular mechanism by which genes are expressed and normally regulated during differentiation using simple eukaryotic model systems. The Office of the Chief, in addition to coordinating the administrative responsibilities of the Laboratory and its sections, conducts research to investigate the molecular structure and function and biochemical properties of select oncogenes, including the ras family of oncogenes and the p21 ras onco-protein(s), as well as the ets genes. Such studies are directed towards a well-defined molecular and biochemical description of malignant transformation by select oncogenes and their products, as well as of their normal cellular counterparts in differentiative and proliferative stages of the cell cycle.

The major portion of the present and future emphases of this Laboratory concerns the identification, isolation and analysis of oncogenic sequences by molecular cloning techniques, as well as their oncogene protein products, in order to evaluate their relationship to the malignant transformation process. We have pursued studies into several major areas. We are analyzing the structural and biological properties of specific retroviral onc genes present in both avian and mammalian acute transforming retroviruses. We are identifying, isolating and characterizing their normal cellular homologs, the proto-onc genes, from their species of origin, as well as from the human genome. Through combined efforts of the sections within the Laboratory of Molecular Oncology, we have developed strategies and protocols to address the mechanisms by which cellular proto-oncogenes can become transduced, activated and expressed, and have attempted to delineate the pleiotropic molecular changes effected by these concerted events. We have also capitalized on our research emanating from investigations performed on acute transforming viruses. In particular, the Carcinogenesis Regulation Section has found that the avian leukemia virus, E26, has homologous sequences in mammalian species which are dispersed to two different chromosomal loci that have distinctive domains; these we have termed ets-1 and ets-2 and they correspond to the 5' and 3' regions of the v-ets oncogene, respectively. In humans these loci have been mapped to chromosome 11 for ets-1, and chromosome 21 for ets-2 by somatic cell hybrid studies and direct in situ analysis using isotopically-labeled probes. In particular, we have, by in situ hybridization of an ets-2 clone to normal human chromosome preparations, confirmed the assignment of human ets-2 to chromosome 21 and regionally

localized it to the HSA 21q22.1-22.3 portion. Similarly, using a characterized panel of mouse and hamster hybrids we have been able to assign the ets-1 and ets-2 to the murine chromosomes 9 and 16, and with the feline hybrids, assigned the proto-oncogenes ets-1 and ets-2 to the feline chromosomes D1 and C2, respectively. Unlike Mammalia, the chicken proto-oncogene for ets was contiguous, indicating that both loci ets-1 and ets-2 are situated on the same chromosome and are not dispersed. From overlapping chicken clones we have found the v-ets oncogene-related exons are dispersed over 35 kb of the avian genomic DNA. From sequenced portions of the human ets-1 and ets-2 clones we have demonstrated an extremely strong (over 90%) homology of predicted amino acid residues compared to the avian and viral oncogenes. The murine ets-2 is essentially identical to the proto-ets-2 gene of humans. The ets-2 homology between humans and Drosophila, as well as humans and sea urchin is also in excess of 90% at the predicted amino acid level. The high levels of amino acid homology observed for the ets genes, the highest thus far noted for any proto-oncogenes, suggest that these genes--from widely separated, evolutionarily-diverse species--must perform important functions to be so stringently conserved. The two genetically distinct loci are transcriptionally and differentially active in human cells, yielding distinct products; they also appear to be independently regulated. We have prepared synthetic oligopeptides corresponding to a conserved predicted amino acid sequence in the ets-2 gene and prepared monoclonal and polyclonal antibodies against this synthetic peptide. Such antipeptide antibodies are able to specifically immunoprecipitate p135gag-myb-ets protein from E26-infected cells. In addition, at least one of these antibodies precipitates a p56 ets-2 protein from human cells.

The anti-oligopeptide antibody to the second human exon proto-oncogene, c-myc, was synthesized by the same strategy used for the ets immune reagent and was shown to react with native c-myc proteins of various human cell lines. This antibody also recognized a protein expressed by cloned human c-myc genes in eukaryotic cells, substantiating its specificity to the c-myc locus. Besides c-myc proteins, the antibody also recognized another protein (80K) which showed a limited similarity with the c-myc proteins in staphylococcal V8 protease partial cleavage mapping. The possible relationship of this protein to the c-myc products is under investigation. In the course of pulse-labeling and subsequent chase of human colon carcinoma cells, four unique polypeptides have been detected by the monoclonal anti-myc sera, suggesting they possibly represent different species of c-myc protein that may be interrelated. Using standard subcellular fractionation and immunoprecipitation techniques we find that the p58 myc is exclusively nuclear, and the p60 myc is distributed between the cytoplasm and nuclei. Additionally, a p80 protein, specifically recognized by the N-terminal, myc-specific monoclonal antibody, is primarily cytoplasmic. The cytoplasmic p60 has been purified to homogeneity and the N-terminal amino acid sequence determined. The amino acid sequence did not correspond to the human c-myc sequence, but does, instead, demonstrate a weak (30-50%) homology to a 5' domain of the human N-myc protein. This finding suggests that myc products may be a member of a gene family with several members recognized by our monoclonal antibody.

The use of a polyclonal antibody prepared against a highly-conserved region of the ets-2 (a region found in all three ets-2 transcripts) identified the p56 protein from a human cell line and in the mouse thymus. Similarly, polyclonal

antibodies prepared against the v-ets-2-expressed protein also immunoprecipitate a p56 protein; the one-dimensional peptide maps of the p56 proteins detected with the anti-peptide antibody and the antibody against the v-ets-expressed protein are exactly identical. Using standard subcellular fractionation techniques, immunoblotting and immunoprecipitation techniques, we have found, using our antibody, that the ets-2 p56 is localized in the nucleus. Our investigations have allowed us to localize the ets genes relative to a number of chromosome breakpoints characteristic of specific translocations occurring in neoplastic cells. We have found the ets-1 gene locates on a very narrow region of chromosome 11, between the breakpoints of the t(4;11)(q21;q23) of an anti-leukemia and the t(11;22)(q24;q12) of an Ewing's sarcoma. The ets-2 gene on chromosome 21 is inactivated by the breakpoints of a t(8;21)(q22;q22) of an acute myelogenous leukemia (AML-M2) and a t(21;22)(q22;q11) of a chronic myeloid leukemia. Both ets-1 and ets-2 were not found to be rearranged using probes representing the 3' regions of the genes.

A cDNA library was prepared from human carcinoma cells; from these, five nearly full-length cDNA clones were isolated that reacted with the human ets-1 genomic probe. Additionally, five recombinant clones specific to human ets-2 cDNA probes have been identified. Two of the five ets-1 cDNA clones were derived from the same class of mRNA as determined by size and restriction map analysis. These findings suggest the existence of multiple transcripts of the ets-1 gene, which may be due to an alternative splicing mechanism. The human ets-2 cDNA clones analyzed fall into two categories of mRNA; based on size and restriction map analysis, each shares a common 5' region. There is also preliminary evidence for an alternative splicing event occurring with the ets-2 mRNA.

To characterize the MH2 virus containing the dual oncogenes, mht and myc, and to elucidate the nature and functional contributions of each, we have constructed deletion and frameshift mutants of each of the two MH2 genes, v-mht and v-myc. Studies on these mutants indicated that the v-myc gene transformed avian primary cells *in vitro* by itself, without requiring the second potential oncogene. The v-mht gene did not show detectable transforming ability *in vitro* but may enhance transformation of primary avian cells in cooperation with the v-myc gene. We have also constructed a replication-defective murine retrovirus carrying the v-myb and v-ets oncogenes. DNA sequence analysis verified that the insertion was in frame with the first 34 codons of murine gag p15. This constructed virus (ME26) encodes a murine gag-avian gag-myb-ets fusion protein of approximately 133 kilodaltons. Following cotransfection into NIH3T3 cells with pSV2neo, G418-resistant colonies were selected. Thirteen of 18 isolated colonies were found to contain integrated ME26 sequences. Significantly, recombinant ME26 virus was rescued from the supernatant of transfected cells upon superinfection with a murine helper virus. The rescued ME26 virus could be successfully passed into NIH3T3 cells by infection, without gross alterations of the recombinant genome. In fact, a protein of about 133 kilodaltons was detected in NIH3T3 cells transfected with the ME26 construct. This protein reacted specifically with the anti-avian gag and anti-ets antisera and may represent an authentic murine-avian viral fusion protein.

We have recently determined the DNA sequences of three avian acute transforming retroviruses that contain the v-myc oncogene, as well as the sequence of the chicken proto-myc gene. We have found that there is a codon where, as a result of adjacent mutations, none of the three viral sequences coding for that amino acid residue were observed to be coded for by the cellular proto-myc gene. This position, numbered 61 from the first ATG in chicken exon 2 and its neighbors, are conserved among the proto-myc genes from chicken, human, mouse and fish. Due to the relatively low number of amino acid differences observed between the viral and chicken myc sequences, it is rather unlikely that this event has occurred by chance alone. There would be only one chance in 458 that all three viral sequences contain an amino acid substitution at a common position if these substitutions had occurred randomly. Substitutions at position 61 may lead to an increase in the oncogenic potential of the virus and, thus, give the transformed cells a selective growth advantage.

Cellular myc sequences from chicken, human, and trout were aligned by computer program. The overall alignment of these three myc sequences contained triple matches at 55% of the total number of positions compared. The pairwise comparisons between the sequences showed homology ranging from 62% matched residues (trout vs. human) to 68% matched residues (chicken vs. human). Smaller regions, however, contain a much higher degree of homology (>80%). Each of these sequences contained short, unique regions that likely were introduced by insertional events.

We have inserted a DNA segment containing 82% of the src open reading frame of HTLV-III/LAV virus into a prokaryotic expression vector, pJL6. The bacterially-synthesized src protein reacted with sera from individuals infected with HTLV-III, indicating that src was expressed as a protein product that was immunogenic in vivo. Antibodies to the purified, bacterially-synthesized src protein immunoprecipitated a 23-kilodalton protein in HTLV-III-infected H9 cells, suggesting that this protein may be the src gene product. In order to determine the cellular location of the src protein, HTLV-III-infected H9 cells were fractionated into cytoplasmic, membrane, and nuclear fractions. The 23-kd protein detected by patient antisera was found to be present only in the cytoplasmic fraction. Several eukaryotic vector constructs were engineered to express the envelope genes of the HTLV-III virus. All such constructs, after cotransfection into TK⁻ cells along with the thymidine kinase gene, gave rise to selective transformants that enabled permanent lines to be established. Eight out of 10 such cell lines were found to be expressing HTLV-III envelope-specific mRNA. Four of these cell lines were expressing very high levels of message specific for the HTLV-III envelope gene. Recombinant vectors containing metallothionein promoter inducible HTLV-III envelope genes have recently been constructed containing SV40 T-antigen splice and polyadenylation signals; they are being introduced into embryonic mice for transgenic expression studies.

Laboratory work in the Office of the Chief, LMO, has focused efforts on understanding the functional roles that oncogenes may play in normal and malignant systems. One approach has been to examine the role specific proto-oncogenes may play in normal cellular development and growth. Thus, research analysis of c-ets gene expression during spermatogenesis, thymus development and during compensatory growth of liver indicates that (i) ets-2 gene expression is linked to cell proliferation and occurs before DNA synthesis, (ii) ets-2 gene

expression may be regulated during the course of development, (iii) both ets-1 and ets-2 genes are differentially regulated, and (iv) ets genes may belong to the nuclear family of oncogenes. In mice, the ets-2 gene is transcribed as a major mRNA species of 4.2 kb and expressed in most of the tissues examined. The ets-1 gene is transcribed as multiple mRNA species sized 7.5 kb, 2.4 kb and 1.7 kb. The product of ets-2 appears to be preferentially expressed as a protein of 56-Kd in size, and has been identified as a putative ets-2 gene protein which is expressed at much higher levels in the thymus. An important role of ets gene products in cell proliferation and differentiation is suggested by these investigations and by the highly conserved nature of this proto-oncogene. Along similar lines the proto-oncogene, c-ets-2, of sea urchin (Lytechinus variegatus) has been molecularly cloned and sequenced. A comparison of the sequence of the c-ets-2 gene of sea urchin was made with the chicken retroviral homolog, v-ets, and the human cellular oncogene, Hu-ets-2. A remarkable conservation of these genes was noted; over 92% of the predicted amino acids of the sea urchin c-ets-2 was homologous to the viral oncogene. More than 94% of the predicted amino acids of sea urchin c-ets-2 matched with the human homolog Hu-ets-2, the highest homology thus far noted for such evolutionarily, widely-distributed oncogenes. The expression of messenger RNA was examined during sea urchin embryogenesis using our cloned c-ets-2 DNA as a probe. It appears that the expression of the sea urchin c-ets-2 gene occurs early during embryonic development, peaking from 5 min to 17 hr post-fertilization, and declining markedly thereafter. The sea urchin mRNA identified by the cloned c-ets-2 probe was a unique species sized at 5.7 kb.

To study myc genes under inducible control we have cloned human myc genes containing entire coding sequences or only the second and third exons and expressed them under the control of the metallothionein promoter, using a BPV vector system. Permanent cell lines expressing human myc proteins have been established. Analysis of human myc gene products in these cell lines indicates that (i) myc gene products enhance BPV-induced transformation, (ii) 62- to 64-Kd human myc protein is made either when all three exons are present or only the second and third exons are present, (iii) human myc protein expressed in mouse cells is mainly compartmentalized in the nucleus, (iv) human myc protein is inducible with heavy metal ions, and (v) though the myc gene is present on an episome in the cell, it appears to be subject to a similar regulatory control mechanism(s) like those controlling the endogenous c-myc gene.

Work in the Office of the Chief has utilized oligonucleotide-directed, site-specific mutagenesis to dissect the biochemical basis of oncogenic activation and enzymatic activity of the ras oncogene. Towards this end, we have constructed several point mutations in the ras gene at the GTP binding site of p21. Both lysine and tyrosine mutations of asparagine-116 abolish GTP binding and transforming activities of p21. These activities are, however, retained by mutations at position 117 or 118. Both position 116 mutant p21s, when overproduced in E. coli, are apparently devoid of GTP binding and autokinase activities. DNA from these mutants does not transform NIH3T3 cells, and cells transfected with these mutants incorporate the exogenous v-ras sequences, express p21, and are contact-inhibited. In contrast to competent clones, defective p21 proteins are not autophosphorylated in vivo, indicating a loss of biochemical activity. Mutations in the ras gene at the glycine coding residues within the ATP/GTP-binding consensus sequences, GXXXGK, also greatly

affected the GTP binding activity. These studies indicate that the GTP binding domain of p21 is critical for its cellular functioning. Further work in this section has precisely characterized functional domains of p21 proteins. We have chemically mapped the palmitoylation site of p21 to cysteine-186, which appears to be located in the membrane binding domain. The GTP binding domain has been defined by chemical, immunochemical and site-directed mutagenesis approaches. The structure of the GTP binding domain is very similar to that of the EF-Tu, the *E. coli* elongation factor. The functional role of p21 in the adenylate cyclase system was studied using the S49 lymphoma cells lacking the regulatory G proteins. These results indicate that the p21 protein does not function directly in the adenylate cyclase system. Chemical reactivity of the sulfhydryl group of p21 was also studied by a thiol-specific reagent, N-ethylmaleimide. A rapid method for detection of ras-transformed cells by flow cytometry was developed. Another immunocytochemical method was developed for assessing p21 in fixed tissue sections. Revertants of Ha-MuSV-transformed MDCK cells were isolated and characterized and p21 was found to possess a more normal phenotype. Studies in progress involve the role of protein kinase C in p21 function, identification of p21 palmitoylation enzymes, and the roles of ras oncogenes in mammary carcinomas of human patients.

In the Microbiology Section we have detected two transfectable transforming sequences following the screening of DNA from a human ovarian carcinoma cell line, OVCAR3, in the nude mouse tumor assay. The morphologically-distinguishable transforming activities show differential response to the glucocorticoid, dexamethasone (DEX), which abolishes the ability to detect morphological transformation induced by one of the two sequences. We have cloned a conserved alu + 10.5-kb fragment from this DEX-sensitive transformant. It represents the fusion of two normally unlinked sequences which apparently occurred during transfection. We have constructed a murine retrovirus (ME26) which carries avian gag, as well as v-myb and v-ets sequences, derived from the avian erythro-leukemia virus, E26. The virus replicates to high titer when rescued by repli-cating murine leukemia virus, and cells infected with the virus express a 140-Kd fusion protein which reacts with anti-avian gag and anti-ets antisera. The virus does not appear to morphologically transform NIH3T3 mouse embryo fibro-blasts. We have transfected a BPV-based construct which contains the human metallothionein gene and the murine sarcoma virus mos gene, either fused to human growth hormone (HGH) sequences under metallothionein control, or fused directly to a metallothionein promoter. Both constructs appear to transform mouse C127 cells and NIH3T3 cells. Very high levels of mos fusion protein can be detected in the presence of cadmium only in cells transformed by the HGH fusion construct.

The Molecular Control and Genetics Section has been focusing its efforts on understanding some of the mechanisms involved in gene expression and control at several levels: transcription initiation, transcription termination and anti-termination and post-transcriptional RNA processing and mRNA decay. Members of this section have extensively characterized the phenomenon termed retroregulation of the λ int expression by RNaseIII. Also, they have cloned, using λ and pBR322 vectors, the rnc gene, encoding RNaseIII from the genomic *E. coli* library. To study RNaseIII's own gene regulation, members of the Molecular Control and Genetics Section have made specific antibodies to RNaseIII and are employing this reagent to quantitate the precise levels of this gene product in cells.

Transcriptional activities of specific genes are being measured both by gene fusions to the lacZ gene and directly by mRNA labeling and hybridization studies.

In the clones of rnc a second gene, era, has been detected in the same operon. Sequence analysis indicates a relationship of this E. coli protein to the yeast ras protein. A complete genetic map of the rnc era region of the coli chromosome has been nearly completed using transposition by mini-tn10 and PI transduction to localize rnc era and its nearby genetic markers: glyA, pdx, nadB and purI. Members of this section have also performed the cloning and insertion of the gene for chloramphenicol acetyl-transferase into the middle of era on a λ vector. This mutant era cannot be transferred to the chromosome to replace the normal gene, indicating that the ras homologous gene, era, is essential to E. coli.

The simple eukaryotic cellular model system being studied by the Molecular Control and Genetics Section is that of Dictyostelium discoideum, which undergoes true multicellular differentiation. As such, it is being used to study the mechanisms which control developmental gene activation during normal differentiation. Because of the similarity of basic regulatory mechanisms, this system represents an attractive alternative to more complex animal research systems. During growth and the early stages of aggregation Dictyostelium cells express 50-55% of their single copy genome as mRNA and HnRNA. An additional 26% of the single copy genome is expressed only during the late stages of development. Initiation of transcription on the late portion of the genome requires cell-cell interaction and cAMP. Because such a high proportion of this small eukaryotic genome is either constitutively transcribed or developmentally induced, it offers a unique opportunity to study the structural organization in chromatin of transcriptionally active genes. Our results indicate that both the constitutively-expressed and developmentally-inducible genes are in a DNaseI-sensitive, active structure in chromatin, regardless if its developmentally-inducible genes are being transcribed. By contrast, micrococcal nuclease is able to identify a structural organization, unique to genes, that are actually in the process of being transcribed. Properties of this organization have been used to resolve oligonucleosomes specifically derived from actively-transcribed genes and to determine their protein composition. Nucleosomes from transcriptionally-active genes are found to be devoid of histone H1, while those from inactive genes contained H1 protein. It would appear from these studies that active genes in growing Dictyostelium cells are present in nucleosomes which have lost histone H1 and contain nonintegral length DNA fragments.

Members of the Cellular Transformation Section have studied retroviruses containing the myc oncogene as part of their genomes which may encode proteins containing both viral structural and cellular oncogene domains (MC29 and CM11 viruses) or only the cellular domain (MC2 and OK10 viruses). They have found that the hybrid protein encoded by MC29 virus, p110^{gag-myc}, migrates to the nucleus soon after synthesis, and can be found associated with the nuclear matrix and with chromatin. This protein has a short half-life (30-40 min) inside cells, and another labile intranuclear protein is responsible for its degradation. About one-third of newly-radiolabeled p110 associates with chromatin, and is more resistant to degradation than the remaining two-thirds,

found free, in nucleoplasm. Preliminary experiments suggest that the association of p110 with chromatin requires binding to DNA, which is consistent with the reported DNA binding ability of p110.

Cells transformed by MC29 virus and related myc-containing viruses have enlarged nucleoli; this distortion is not a consistent feature of other transformed or nontransformed cells. These myc-transformed cells were found to incorporate a high proportion of radioactive uridine into nucleolar RNA. Also, transcription in isolated nuclei was more resistant to a specific inhibitor of mRNA synthesis, demonstrating an enhanced level of rRNA synthesis. Failure of rRNA processing enzymes to keep pace with increased synthesis resulted in the accumulation of an rRNA precursor (32S RNA), which was the main RNA constituent of the enlarged nucleoli of myc-transformed cells. Analysis of nucleolar proteins demonstrated the increased synthesis of a specific protein, p98, which may be responsible for the noted increased rRNA synthesis. The apparent stimulation of p98 synthesis may be related to the presence of v-myc indirectly, in spite of the fact that the p110^{9a9}-myc protein was not found in purified nucleoli.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01CP04876-14 LMO

PERIOD COVERED

October 1, 1985 to September 30, 1986

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Oncogenic Virus Influence on the Biochemical Events of Host Cells

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: P. S. Ebert Chemist LMO NCI

Other: J. P. Bader Research Microbiologist LMO NCI

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Molecular Oncology

SECTION

Cellular Transformation Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701-1013

TOTAL MAN-YEARS:

1.0

PROFESSIONAL:

1.0

OTHER:

0.0

CHECK APPROPRIATE BOX(ES)

- (a) Human subjects (b) Human tissues (c) Neither
- (a1) Minors
- (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The v-myc protein, p110, produced by MC29 nonproducer quail cells (Q8) was substantially purified by sequential column chromatography. The most efficient purification was achieved by a sequence of Sephacryl S-400, DEAE-Sephadex, and G-75 columns. Stages of purification were monitored by immunoprecipitation of the p110 with gag and myc antisera. Potassium-adenosine triphosphatase (ATPase) was found to be associated with the p110 throughout the purification. The enzyme activity could not be eliminated by high speed centrifugation or sonication. The purified p110-ATPase showed optimum activity at pH 6.0 and in the absence of Mg⁺⁺. The p110 did not show any in vitro adenylation activity.

PROJECT DESCRIPTIONNames, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

P. S. Ebert	Chemist	LMO NCI
J. P. Bader	Research Microbiologist	LMO NCI

Objectives:

To determine the biological function and enzymatic activities of proteins expressed by the myc oncogene.

Methods Employed:

Adenosine triphosphatase (ATPase) was measured by incubating column fractions and staphylococcal protein A sepharose-bound immune complexes with an incubation mixture containing λ - ^{32}P -labeled ATP. Following adsorption of unreacted ATP onto activated charcoal, the labeled phosphate released was measured by scintillation counting. MC29 nonproducer quail cells (Q8) which express the v-myc protein, p110^{gag-myc}, were incubated with ^{35}S -methionine to label the nuclear p110. The nuclei were extracted from Q8 cells with detergent and partially purified by centrifugation through 0.9 M sucrose. The nuclei were sonicated to solubilize the p110, and the ^{35}S -labeled protein was passed through chromatofocusing, anion exchange (DEAE-Sephadex) and various molecular sieving columns (S-400, S-500 and G-75). The presence of p110 at each phase of purification was verified by immunoprecipitating the p110 in the column fractions with various gag and myc antibodies. Labeled protein bands were visualized by subjecting the concentrated fractions to polyacrylamide gel electrophoresis and exposing the dried gels to X-ray film. Since the SV40 large tumor antigen exhibits an in vitro adenylation activity, attempts were made to determine if similar activity could be detected in the myc protein. Possible adenylation activity of the p110 was investigated by incubation of Q8 nuclear extracts in vitro with α - ^{32}P -ATP and examining polyacrylamide gels for p110 labeling.

Major Findings:

p110 was substantially purified from Q8 nuclei by sequential column chromatography so that this species appeared as the major labeled band by PAGE gel chromatography. Column chromatography at 4° was utilized to resolve the p110 and preserve any possible enzymatic activity associated with it. The most efficient purification was achieved by a sequence of S-400, DEAE-Sephadex, and G-75 columns. The latter step removes most of the cellular proteins of molecular weights below 80,000. However, the greater amount of the isolated pool of p110 was contaminated with p60 and p75 bands of proteins which co-migrated with the p110 throughout the purification sequence. The purified p110 was immune-precipitable by gag and myc antisera. The tendency of p110 to aggregate could be reduced by the addition of dithiothreitol to the fractions and storing the samples at 4°. A K-ATPase co-migrated with the p110 throughout the triple column sequence. This enzyme activity could not be removed by two 100,000 x g centrifugations and two intervals of sonication. The purified p110-ATPase

showed different optimal incubation conditions from that enzyme associated with α -gag immunoprecipitates in that the purified ATPase required no Mg^{++} and a pH of 6.0. Attempts to detect an in vitro adenylation activity in v-myc protein were unsuccessful.

Publications:

None

Patents:

None

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01CP04899-14 LMO

PERIOD COVERED

October 1, 1985 to September 30, 1986

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Transforming Genes of Avian RNA Tumor Viruses

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	I. S. Papas	Chief	LMO	NCI
Others:	R. J. Fisher	Expert	LMO	NCI
	J. A. Lautenberger	Senior Staff Fellow	LMO	NCI
	D. K. Watson	Senior Staff Fellow	LMO	NCI
	N. C. Kan	Senior Staff Fellow	LMO	NCI
	N. Sacchi	Visiting Associate	LMO	NCI
	N. K. Bhat	Visiting Fellow	LMO	NCI
	S. Fujiwara	Visiting Fellow	LMO	NCI

COOPERATING UNITS (if any)

Department of Biology, Johns Hopkins University School of Medicine, Baltimore, MD (G. Scangos and E. Moudrianakis); Department of Biology, University of California, Berkeley, CA (P. Duesberg)

LAB/BRANCH

Laboratory of Molecular Oncology

SECTION

Carcinogenesis Regulation Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701-1013

TOTAL MAN-YEARS:

1.0

PROFESSIONAL:

1.0

OTHER

0.0

CHECK APPROPRIATE BOX(ES)

- (a) Human subjects (b) Human tissues (c) Neither
- (a1) Minors
- (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

A major effort of the LMO is to elucidate the processes by which specific retroviral oncogenes, as well as their cellular homologs, are able to impact on critical cellular events. The oncogene elements, derived from avian leukemia viruses, have been intensively investigated and analyzed to define their structural and genomic organization. Using the oncogenes myc, mht and ets as probes, we have detected, isolated and cloned the cellular homologs of these genes from evolutionarily diverse organisms such as humans, mice, cats, fish, sea urchin and Drosophila. Specific regions of these cellular genes are retained at high levels of homology and each was compared and examined, as was its expressed product. In all cases, the proto-oncogenes were significantly larger than their corresponding viral oncogenes. This consistent truncation of the viral oncogene and its products may implicate this as a general mechanism in the critical events controlled and regulated by these highly conserved genes. We have also developed and exploited several expression vector systems, both prokaryotic and eukaryotic, to produce oncogene-specific products in quantity. These products were used to purify, characterize and develop immunologic reagents to locate the cellular proto-oncogene products, and characterize their molecular structure. Such reagents have also been used to probe for the expression of oncogene-specific products in normal and malignant tissues and related them to specific human pathologies. In certain leukemias we have noted an alteration in the pattern of expression of the ets genes, compared to the expression in normal blood cells. These studies may have a useful application in the diagnostic and clinical evaluation of malignancy and how the cell progresses into neoplasia.

PROJECT DESCRIPTIONNames, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

T. S. Papas	Chief	LMO	NCI
R. J. Fisher	Expert	LMO	NCI
J. A. Lautenberger	Senior Staff Fellow	LMO	NCI
D. K. Watson	Senior Staff Fellow	LMO	NCI
N. C. Kan	Senior Staff Fellow	LMO	NCI
N. Sacchi	Visiting Associate	LMO	NCI
N. K. Bhat	Visiting Fellow	LMO	NCI
S. Fujiwara	Visiting Fellow	LMO	NCI

Objectives:

To investigate the processes by which retroviruses are able to exert their subversive influences, via genetic expression of their viral oncogenes, to manifest malignant transformation in vitro and neoplastic diseases in vivo. To investigate and determine the molecular interrelationship of viral oncogenes to their normal cellular counterparts, the proto-oncogenes, and to comprehend their structural similarities, differences and functional natures, respectively. To utilize the methods of recombinant DNA technology and monoclonal-hybridoma research in order to identify and understand the role of select viral and cellular oncogenes in the normal and malignant cellular growth and differentiative processes.

Methods Employed:

1. Preparation of high molecular weight nucleic acids from eukaryotic cells was performed by standard methods, as described (Molecular Cloning, A Laboratory Manual, 1982) and modified according to our needs relative to phage, plasmid or prokaryotic systems employed.
2. Nucleic acid analysis by standard techniques described for restriction enzyme analysis, resolution of components by agarose and/or polyacrylamide gel electrophoretic systems; sequencing analysis by Maxam-Gilbert (Methods Enzymol. 65: 499-560, 1980); techniques incorporating the hybridization methods of Southern blot and Northern transfer protocols, as described (Molecular Cloning, A Laboratory Manual, 1982), in conjunction with radioisotopic labeling procedures for preparation of onc-specific, cloned DNA probes by nick-translation using E. coli DNA polymerase and DNaseI.
3. Construction of recombinant phage libraries and plasmids by restriction/ligation of fragments of isolates of eukaryotic DNA homologous to onc genes; subcloning, as required, into pBR322, pBR325 or pBR328 or into cosmid or λ vectors, followed by production of phage by in vitro packaging.
4. Construction of cDNA library: Double-stranded cDNA was prepared from poly A⁺ RNA using cloned reverse transcriptase enzymes and ligated into λ gt10 vectors or bacterial vectors for amplification.

5. Radiolabeling of bacterially-expressed proteins or eukaryotic cellular proteins was performed using ^{35}S -methionine or -cysteine isotopes, followed by cellular fractionation and purification protocols, and subsequent identification of labeled protein products by SDS-PAGE analysis, visualized by autoradiography and/or Western blot (immunoblot electrophoresis) methods.
6. Immunologic analyses were performed using polyclonal or monoclonal antibodies prepared against oligopeptide-specific antigens constructed from the deduced, conserved nucleic acid sequences of specific onc genes or from bacterially-expressed products, purified and used as antigens, and analyzed by immunoprecipitation and Western blot hybridization procedures.
7. Protein, polypeptide and peptide analyses were performed using a combination of SDS-PAGE and HPLC-FPLC chromatographic techniques, in conjunction with specific cleavage procedures employing proteolytic enzymes or controlled chemical hydrolysis methods, on preparative and analytical scales. Confirmation of protein analysis was performed by microsequencing techniques using an automated gas-phase amino acid microsequencer in conjunction with high performance liquid chromatography.

Major Findings:

We have determined the complete nucleotide sequence of the chicken ets gene and compared it to the ets gene of E26. E26 is a genetic hybrid with sequences derived from viral structural genes and parts of essential cellular proto-onc genes. The cellular genes contain additional 5' sequences. The avian cellular gene transcript is considerably larger than the amount of DNA transduced by the virus. We have determined that the mammalian homologs of v-ets consist of two distinct domains located on different chromosomes. We have shown that both loci (ets-1 and ets-2) are transcriptionally active, producing distinctive mRNA species. The sequences homologous to ets-1 and ets-2 are colinear in chicken proto-ets and have possibly become separate and functionally distinct since before the evolutionary divergence of Mammalia. The mammalian ets genes from man and mouse encode essentially identical amino acids, and are over 90% conserved relative to the chicken ets gene.

To further characterize the cellular proto-oncogene products encoded by these oncogenes, particularly in human cells, we have prepared several types of polyclonal or monoclonal antibodies against oligopeptides representing conserved amino acid sequences derived from the myc or ets DNA sequence.

Using these antibodies has enabled the ets-2 and myc proteins to be localized subcellularly. The ets-2 protein shows primarily a nuclear localization, while the myc protein, in addition to showing a nuclear localization, shows also a cytoplasmic localization.

Using these antibodies, we have identified an ets-2 p56 protein and several myc p58, p60 (cytoplasmic) and p80 (cytoplasmic) proteins which have been partially or completely purified (myc p60). The N-terminal amino acid sequence of the myc p60 (cytoplasmic) has been determined and found not to correspond to the derived amino acid sequence of myc. This new protein may thus correspond to a family of myc-related proteins.

A monoclonal antibody also detects a p80 myc-related polypeptide besides other c-myc proteins; its possible relationship to c-myc products is being investigated. In the course of pulse-labeling and subsequent chase of COLO 320 cells, four different myc-related polypeptides have been detected by this antibody. They possibly represent different species of a family of human c-myc proteins. Pulse-chase studies suggest a precursor-product relationship between some of these polypeptides. The proteins recognized by this monoclonal antibody are being purified for amino acid sequencing.

Using ets-specific probes we have found that human ets genes are located on chromosome regions of interest to human cancer, as well as to human genetics. These regions are the 11q23-24 for ets-1 and 21q22.3 for ets-2. Cancer-specific chromosome abnormalities involving these regions are found mainly in leukemias, lymphomas and solid tumors (Ewing's sarcoma). We have localized the ets genes relative to a number of chromosome breakpoints characteristic of specific translocations occurring in neoplastic cells. We have found the ets-1 gene located in a very narrow region of chromosome 11, between the breakpoint of the t(4;11)(q21;q23) of an acute leukemia and the t(11;22)(q24;q12) of a Ewing's sarcoma. The ets-2 gene on chromosome 21 is bracketed by the breakpoints of a t(8;21)(q22;q22) of an acute myelogenous leukemia (AML-M2) and a t(21;22)(q22;q11) of a chronic myeloid leukemia. Both ets-1 and ets-2 were not found to be rearranged. We have found that human ets DNA is located on two different chromosomes. The human ets-1 locus on chromosome 11 encodes a single mRNA of 6.8 kb; the second ets-2 locus, on chromosome 21, encodes three mRNAs of 4.7, 3.2 and 2.7 kb. To study the structural organization and splicing mechanism of the human ets-1 and ets-2 genes, we prepared a cDNA library from human COLO 320 cells which expresses very high levels of ets-specific transcripts. Several recombinant clones reactive with ets-1 and ets-2 probes were isolated. These cDNA clones were characterized by restriction mapping and Southern blot analysis into different families of mRNAs. These clones are also being sequenced; there is preliminary evidence that these multiple transcripts, in the case of ets-1 and ets-2, may have been generated through alternative splicing events.

We have constructed a murine retrovirus (ME26) which carries avian gag, as well as v-myb and v-ets sequences derived from the avian erythroleukemia virus, E26. The virus replicates to high titer when rescued by replicating murine leukemia virus. Cells infected with this virus express a 140-Kd fusion protein which reacts with anti-avian gag and anti-ets antisera. This virus does not appear to transform NIH3T3 mouse embryo fibroblasts.

Comparison of the deduced amino acid sequences of three viral myc sequences (from avian acute leukemia viruses MC29, MH2, and OK10) with cellular proto-myc sequences from trout, chicken, mouse, and human cells indicates that there is a single position where each of the viral sequences differs from the cellular sequences. This position and its neighbors are completely conserved in the vertebrate sequences examined; this unique arrangement is unlikely to have occurred by chance alone.

Analysis of c-ets gene expression during spermatogenesis, thymus development and during compensatory growth of liver indicates that (i) ets-2 gene expression is linked to cell proliferation and occurs before DNA synthesis, (ii) ets-2 gene expression may be regulated during the course of development, (iii) both ets-1

and ets-2 genes are differentially regulated, and (iv) ets genes may belong to the nuclear family of oncogenes. In mice, the ets-2 gene is transcribed as a major mRNA species of 4.2 kb and expressed in most of the tissues examined. The ets-1 gene is transcribed as multiple mRNA species sized 7.5 kb, 2.4 kb and 1.7 kb. The putative product of the ets-2 gene appears to be preferentially expressed as a protein of 56 Kd and is expressed at much higher levels in the thymus.

Human myc genes containing the entire coding sequence, or only the second and third exons, have been expressed under the control of the metallothionein promoter, using the bovine papillomavirus (BPV) vector system. Permanent cell lines expressing human myc proteins have been established. Analysis of human myc gene products in these cell lines indicates that (i) myc gene products enhance BPV-induced transformation, (ii) 62-64-Kd human myc protein is made either when all three exons are present or only when second and third exons are present, (iii) human myc protein expressed in mouse cells is mainly compartmentalized in the nucleus, (iv) human myc protein is inducible with heavy metal ions, and (v) though the myc gene is present on an episome in the cell, it appears to be subject to a similar regulatory control mechanism(s) like those controlling the endogenous c-myc gene.

The proto-oncogene, c-ets-2, of sea urchin (Lytechinus variegatus) has been molecularly cloned and sequenced. A comparison of the sequence of the c-ets-2 gene of sea urchin was made with the chicken retroviral homolog (v-ets) and the human cellular oncogene, Hu-ets-2. A remarkable conservation of these genes was noted; over 92% of the predicted amino acids of the sea urchin c-ets-2 was homologous to the viral oncogene. More than 94% of the predicted amino acids of sea urchin c-ets-2 matched with the human homolog Hu-ets-2, the highest homology thus far noted for such evolutionarily, widely-distributed oncogenes. The expression of messenger RNA was examined during sea urchin embryogenesis, using our cloned c-ets-2 DNA as a probe. It appears that the expression of the sea urchin c-ets-2 gene occurs early during embryonic development, peaking from 5 min to 17 hr post-fertilization, and declining markedly thereafter. The sea urchin mRNA identified by the cloned c-ets-2 probe was a unique species sized at 5.7 kb.

To provide a genetic system to study oncogenes during development, we have successfully isolated and cloned a Drosophila ets gene highly conserved at the amino acid level (over 90%) to the viral ets-2 and human ets-2 sequences. The expression of Drosophila ets-2 during development is variable during different periods of embryonic differentiation.

Publications:

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Patents:

None

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01CP04963-10 LMO

PERIOD COVERED

October 1, 1985 to September 30, 1986

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Toward a Molecular Description of Malignant Transformation by p21 ras Oncogenes

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)

PI:	T. Y. Shih	Research Chemist	LMO	NCI
Others:	L. S. U'ish	Microbiologist	LMO	NCI
	D. J. Clanton	Senior Staff Fellow	LMO	NCI
	D. T. Kiang	Guest Researcher	LMO	NCI
	J. M. Ward	Vet. Medical Officer	LCC	NCI
	H. A. Young	Senior Scientist	BRMP	NCI
	T. S. Papas	Chief	LMO	NCI
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COOPERATING UNITS (if any) LMB, NIADDK, NIH (D. Davies); LCDB, NIADDK, NIH (M. Lin and S. Beckner); ERBB, NICHD, NIH (K. P. Huang); LMVC, LBI, Frederick, MD (S. Oroszlan); U. of Tokyo (S. Hattori); Dept. Biol. Chem., Washington U. School of Medicine (L. Glaser); NAPS, PRI, Frederick, MD (M. Zweig, G. DuBois, S. Showalter)

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Laboratory of Molecular Oncology

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INSTITUTE AND LOCATION

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TOTAL MAN-YEARS:

2.0

PROFESSIONAL:

1.0

OTHER:

1.0

CHECK APPROPRIATE BOX(ES)

- (a) Human subjects (b) Human tissues (c) Neither
- (a1) Minors
- (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The major focus of this project is to investigate the molecular biology and biochemistry of the ras oncogenes and the ras p21 proteins. The long-range goal is to elucidate molecular mechanisms of cell transformation induced by these genes and their protein products, with the purpose of contributing to our understanding of the roles of oncogenes in human tumorigenesis, and to develop strategies for tumor detection, monitoring and intervention. We have characterized the functional domains of p21 proteins. We have chemically mapped the palmitoylation site of p21 to cysteine-186, which appears to be the membrane binding domain. We have characterized the GTP binding domain by chemical, immunochemical and site-directed mutagenesis approaches. The structure of the GTP binding domain is very similar to that of the EF-Tu, the *E. coli* elongation factor, of which a three dimensional structure has recently been determined. The functional role of p21 in the adenylate cyclase system was studied using the S49 lymphoma cells lacking the regulatory G proteins. The results indicate that p21 does not function directly in the adenylate cyclase system. Chemical reactivity of the sulfhydryl group of p21 was also studied by a thiol-specific reagent, N-ethylmaleimide. A rapid method for detection of ras-transformed cells by flow cytometry was developed. Another immunocytochemical method was developed for assessing p21 in fixed tissue sections. Revertants of Ha-MuSV-transformed MDCK cells were isolated and characterized. Studies in progress involve the role of protein kinase C in p21 function, identification of p21 palmitoylation enzymes, and the roles of ras oncogenes in mammary carcinomas of human patients.

PROJECT DESCRIPTIONNames, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

T. Y. Shih	Research Chemist	LMO	NCI
L. S. Ulsh	Microbiologist	LMO	NCI
D. J. Clanton	Senior Staff Fellow	LMO	NCI
D. T. Kiang	Guest Researcher	LMO	NCI
J. M. Ward	Vet. Medical Officer	LCC	NCI
H. A. Young	Senior Scientist	BRMP	NCI
T. S. Papas	Chief	LMO	NCI
J. A. Lautenberger	Senior Staff Fellow	LMO	NCI

Objectives:

The major focus of this project is to investigate the molecular biology and biochemistry of the ras oncogenes and the p21 ras oncogene proteins. The long-range goal is to elucidate molecular mechanisms of cell transformation induced by these genes and their protein products, with the purpose of contributing to our understanding of the roles of oncogenes in human tumorigenesis, and to develop strategies for tumor detection, monitoring and intervention. Earlier work on this project has contributed to the identification of the ras oncogenes and their encoded proteins in Harvey and Kirsten murine sarcoma viruses (J. Virol. 25: 238-252, 1978; Virology 96: 64-79, 1979), and characterization of the major properties of the ras p21 proteins, i. e., activities associated with guanine nucleotide binding (Nature 287: 686-691, 1980). These observations led to the current concept that ras genes probably function as cellular signal transducers relaying growth control signals to the interior cellular effectors in the control mechanisms of cell proliferation. Many other studies have suggested the important roles of ras oncogenes in the development of many human tumors. Recently, with the collaboration of Drs. Papas and Lautenberger, biochemically active ras proteins have been overproduced in E. coli, yielding a major research avenue to elucidate in detail the structure and function of p21 proteins (Science 221: 858-860, 1983). The major emphasis of the current study is to investigate the structure-function relationship of the ras proteins in order to elucidate their roles in the control mechanisms of cell proliferation, and to understand the mechanisms of proto-oncogene activation.

Methods Employed:

1. Purification of p21 from E. coli. The E. coli-carrying plasmid, pJLcIrasI, was induced by raising the temperature from 31° to 41°C to overproduce p21 proteins. p21 was purified to over 95% under nondenaturing conditions for enzymology studies. For some less soluble mutant proteins, an alternative procedure was used, which involves cell lysis with lysozyme and NP40, and after centrifugation, extraction of p21 from pellets with 8 M urea.

2. Guanine nucleotide binding assays. The standard assay involves incubation of p21 with ³H-GDP, and retention of the binary complexes with nitrocellulose filters. An alternative assay was performed by Western blot transfer of p21.

3. Autokinase and GTPase assays. These were performed by incubating p21 with gamma-³²P-GTP, and detection of either phosphorylated p21 or released P_i.
4. Monoclonal antibodies to p21. These antibodies were prepared by immunizing mice with purified p21 isolated from E. coli.
5. Site-directed mutagenesis. The ras oncogene of the proviral pH-1 DNA clone was mutagenized by oligonucleotides 17 bases long with single base changes from the wild-type Ha-ras genes. Mutant ras genes were either reconstructed into the pH-1 clones for transfection assays, or inserted into the pJL6 vector for expression of mutant proteins in E. coli.
6. Transfection of NIH3T3 cells. Focus-forming assays were performed by the calcium phosphate precipitation method. A positive selection marker, pSV2-neo, was used for cloning of cells resistant to G418.
7. Immunoprecipitation of p21. Cells were labeled with ³⁵S-methionine or -cysteine, or with ³²P-orthophosphate, and p21 was immunoprecipitated by monoclonal antibodies.
8. Peptide chemistry of p21. Peptide mapping after trypsin digestion was performed by thin-layer chromatography or by high performance liquid chromatography. Peptides were synthesized by solid phase methods.

Major Findings:

1. Functional domains of p21 ras proteins. p21 proteins contain at least two functional domains. The membrane binding domain apparently involves a short sequence at the C-terminus of p21. We have determined by chemical methods that cysteine-186, four amino acid residues from the end, is the palmitoylation site. We have studied a highly purified p21 protein overproduced in E. coli. Scatchard analysis and probing with monoclonal antibodies against p21 indicate a single site per molecule for binding GTP or GDP with a K_d of approximately 10 nM. The same site apparently is involved with the autokinase and GTPase activities of p21. We have constructed point mutations in order to dissect the structure-function relationship of the GTP binding domain. Mutations at asparagine-116 of p21 to lysine or tyrosine, but not at the adjacent 117th or 118th positions, abolish GTP binding activity. Mutations of the autophosphorylation site at threonine-59 to a serine or an alanine residue reduces or abolishes the autokinase activity, consistent with juxtaposition of this residue to the GTP gamma-phosphate. Mutations of glycine residues to valine of the consensus sequence, GXXXXGK, of the p21 N-terminal regions also greatly affect the GTP binding activity. These studies suggest that the basic structure of the GTP binding domain is very similar between p21 and EF-Tu, the E. coli elongation factor, of which a three dimensional structure has recently been determined by other investigators. Transfection studies of the mutant ras genes indicate that the GTP binding domain is crucial for p21 cellular function.
2. Functional roles of p21 in cellular signal transduction. By reconstitution with a membrane preparation of the S49 lymphoma cell line lacking the N_s protein of adenylate cyclase with purified p21, no adenylate cyclase activity can be

restored, suggesting that p21 does not function as N_5 . No ADP ribosylation has been found for purified p21 or p21 in situ in plasma membrane. p21 also does not function as N_1 , since there is no change in either basal- or foreskin-stimulated adenylate cyclase activity with purified p21. These results suggest that the immediate target of p21 in cellular growth signal transduction is not adenylate cyclase, an observation different from the marked effect seen in some yeast strains.

3. Chemical probes on the dynamic structure of p21. As an approach to a probe for the possible conformational changes of p21 associated with ligand bindings or target interactions, we have studied the sensitivity of sulfhydryl groups of a highly purified p21 protein with a thiol specific reagent, N-ethylmaleimide (NEM). Approximately 70% of GTP binding and autokinase activities of p21 were inactivated by NEM and excessive amounts of GTP or GDP protected p21 activities. This GTP-modulated sulfhydryl group was identified as cysteine-80 by comparative peptide mapping of ^{14}C -NEM modified p21 in the presence and absence of GTP. This region of p21 shares an extensive sequence homology with various G proteins, and appears to be in the vicinity of the GTP binding domain of these proteins.

4. Detection of the intracellular ras p21 by flow cytometry. Rapid identification of the expression of oncogene products in specific cell types could potentially be useful in the diagnosis and treatment of human malignancy. We have now observed that through the use of lysolecithin permeabilization and fluorescence activated flow cytometry, cells expressing high levels of p21 can be readily distinguished from the nontransformed parent cells in a rapid and quantitative manner.

5. Immunocytochemical localization of p21 in fixed tissue sections of mice. Short-term fixation in Bouin's fixative was found to be the most effective method for demonstrating p21 in fixed tissue sections. Ha-v-ras p21 was found in 5-80% of the Ha-MuSV induced sarcoma cells, depending on the tissue fixative and antibody dilution. Splenic erythroblasts in Ha-MuSV induced erythroblastosis contained membrane antigen, as did some reticular cells in lymph nodes draining the sarcomas. Normal tissues were all negative. The degree of immunoreactivity was related to the expected level of p21 expression.

6. Revertants of Ha-MuSV-transformed MDCK cells express reduced levels of p21 and possess a more normal phenotype. Four subclones of MDCK cells transformed by Ha-MuSV have been isolated. These subclones fall into two classes. Two subclones have a fibroblastic morphology, have lost the growth requirement for prostaglandin E_1 (PGE_1), do not respond to glucagon or vasopressin, and in general appear transformed. Two other subclones have epithelioid morphology, are growth-stimulated by PGE_1 , and respond to vasopressin with an increase in intracellular cAMP. We propose that these cells represent revertants to a more nontransformed phenotype. Unlike normal cells, however, these revertants grow under anchorage-independent conditions, express detectable but reduced amounts of v-ras p21, and grow in nude mice. The appearance of such revertants may be one cause of the observed heterogeneity of tumor cells.

7. Protein kinase C (PKC) in p21 function. Purified p21 was found to be phosphorylated by PKC. Work is in progress to delineate the phosphorylation sites and to elucidate the role of PKC in p21 function.

8. The enzymatic mechanism of p21 palmitoylation. Acylation of p21 is essential for p21 cellular activities. An enzymatic activity for p21 acylation has been detected in rat liver microsome fractions using palmitoyl-CoA as the acyl donor. Collaborative work with Prof. Luis Glaser at the Washington University School of Medicine is in progress to identify and characterize this synthetase in yeast extracts.

9. The roles of ras oncogenes in human mammary carcinomas. In collaboration with Prof. David Kiang at the University of Minnesota School of Medicine, we are exploring the roles of p21 ras proteins in hormone-dependent or -independent mammary carcinomas in human patients.

Publications:

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Patents:

None

NOTICE OF INTRAMURAL RESEARCH PROJECT

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PERIOD COVERED

October 1, 1985 to September 30, 1986

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Biochemistry of Cellular Transformation by Avian Tumor Viruses

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)

PI:	J. P. Bader	Research Microbiologist	LMO	NCI
Others:	D. A. Ray	Chemist	LMO	NCI
	F. A. Hausman	Chemist	LMO	NCI

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Laboratory of Molecular Oncology

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2.8

PROFESSIONAL:

1.0

OTHER:

1.8

CHECK APPROPRIATE BOX(ES)

- (a) Human subjects (b) Human tissues (c) Neither
- (a1) Minors
- (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Retroviruses containing the myc oncogene as part of their genomes may encode proteins containing both viral structural and cellular oncogene domains (MC29 and CMII viruses), or only the cellular domain (MH2 and OK10 viruses). The hybrid protein encoded by MC29 virus, p110 gag-myc, migrates to the nucleus soon after synthesis, and can be found associated with the nuclear matrix and with chromatin. The protein has a short half-life (30-40 min) in the cells, and another labile intranuclear protein has been shown to be responsible for its degradation. About one-third of newly radiolabeled p110 is associated with chromatin, which is more resistant to degradation than the two-thirds found free in nucleoplasm. Preliminary experiments suggest that the association of p110 with chromatin requires binding to DNA, which is consistent with the reported DNA-binding capability of p110.

Cells transformed by MC29 virus and related viruses have enlarged nucleoli which are not a consistent feature of other transformed or nontransformed cells. These myc-transformed cells incorporated a high proportion of radioactive uridine into nucleolar RNA. Also, transcription in isolated nuclei was more resistant to a specific inhibitor of mRNA synthesis, demonstrating an enhanced level of rRNA synthesis. Failure of rRNA processing enzymes to keep pace with increased synthesis resulted in the accumulation of an rRNA precursor (32S RNA), which was the main RNA constituent of the enlarged nucleoli of myc-transformed cells. Analysis of nucleolar proteins demonstrated the increased synthesis of a specific protein, p98, which may be responsible for the noted increased rRNA synthesis. The apparent stimulation of p98 synthesis by myc proteins is under investigation.

PROJECT DESCRIPTIONNames, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

J. P. Bader	Research Microbiologist	LMO	NCI
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F. A. Hausman	Chemist	LMO	NCI

Objectives:

To determine the intracellular location and stability of proteins encoded by the avian MC29 virus and related viruses, and of normal cellular proteins homologous to these viral proteins. To analyze the structure of these proteins and their association with cellular constituents. To analyze the factors responsible for enlargement of nucleoli in cells transformed by MC29 and related viruses.

Methods Employed:

Nontransformed cells and cells transformed by avian viruses were cultured in the laboratory, and observed microscopically for morphological differences. Unlabeled cells and cells labeled with radioactive precursors were disrupted and various organelles and other fractions were isolated and subjected to biophysical or biochemical analyses, including (1) quantitative chemical determinations of protein, RNA, DNA, and various enzymatic activities; (2) immunoreactions; (3) sieving and ion exchange chromatography; (4) density gradients, zonal centrifugation and analysis; (5) polyacrylamide and agarose gel electrophoresis, and autoradiography and fluorography of such gels; (6) synthesis of macromolecules in isolated organelles; (7) chemical and enzymatic treatments of proteins and nucleic acids.

Major Findings:

The synthesis, stability, and localization of the protein responsible for transformation by MC29 virus were examined by radioactive labeling of transformed cells, and immunoselection of the virus-coded p110. The p110 migrated rapidly to the nucleus after synthesis, and sequential extractions revealed that it was associated with a fraction considered the nuclear matrix. The p110 under natural conditions decayed with a half-life of 30-40 minutes, but inhibition of RNA synthesis subsequent to labeling extended the half-life. When protein synthesis was inhibited for several hours, then released, newly synthesized p110 was stabilized by further inhibition of protein synthesis. Also, when nuclei were isolated from pulse-labeled cells, the p110 degraded at a reduced rate compared to intact cells. We concluded from these and other studies that another labile protein is responsible for the degradation of p110.

Analysis of disrupted nuclei showed that about one-third of newly radiolabeled p110 was associated with chromatin, with the remaining two-thirds in the nucleoplasmic fraction. The chromatin-associated p110 was considerably more stable than that in the nucleoplasm, and stability was increased by addition of

inhibitors of RNA synthesis after labeling. On the other hand, pretreatment of cells with a DNA-binding antimetabolite prevented the association of p110 with chromatin. Also, a high proportion of chromatin-associated p110 was phosphorylated compared to p110 in the nucleoplasm. The results suggest that phosphorylated p110 associates with DNA in chromatin, and is dissociated in the process of transcription. The particular sites of association are under investigation.

Previous experiments in this lab had demonstrated that p110 occurred in monomeric and dimeric forms. When analyzing these forms using sieving columns with large retention size, we found p110 associated with nucleic acids. Similarly, immunoprecipitation of p110 from nondenatured cellular extracts selected nucleic acids along with p110. We are attempting to identify these nucleic acids in attempts to more specifically localize the function of p110, and to determine the relevance of monomeric and dimeric forms to myc protein function.

Avian cells transformed by MC29 and related viruses containing the myc gene exhibit enlarged nucleoli compared to nontransformed cells or cells transformed by other avian viruses. The size and integrity of nucleoli in these cells were directly dependent on continued RNA synthesis, and addition of exogenous uridine to cells resulted in a relatively greater incorporation into nucleolar RNA than into the rest of the cellular RNA. Nuclei were isolated from myc-transformed and other cells, and transcription was analyzed directly after addition of ribonucleotide triphosphates. Using the drug, amanitin, an inhibitor of other types of RNA synthesis, showed that transcription of ribosomal genes was proportionately greater in myc-transformed cells. Time course experiments examining the rate of processing of ribosomal RNA (rRNA) precursors revealed that 32S RNA accumulated in nucleoli, presumably the rate-limiting step in rRNA processing in these cells. We concluded that myc-transformed cells synthesize pre-rRNA at a disproportionate rate, and that the processing mechanism of this RNA cannot keep pace with the increased synthesis, resulting in the accumulation of 32S RNA and enlarged nucleoli.

A comparison of nucleolar proteins extracted from myc-transformed and other cells showed both the increased synthesis and accumulation of a particular protein, p98. No p110 gag-myc was found in purified nucleoli of MC29 cells. The nucleolar p98 had a half-life of over 4 hr, and was phosphorylated. Other properties of this prominent protein are under investigation.

Publications:

Bader, J. P., Hausman, F. A. and Ray, D. A.: Intranuclear degradation of the transformation-inducing protein encoded by avian MC29 virus. J. Biol. Chem. (In Press)

Bader, J. P., Ray, D. A. and Hausman, F. A.: On the enlarged nucleoli of cells transformed by myc-containing viruses. Mol. Cell. Biol. (In Press)

Patents:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05120-07 LMO

PERIOD COVERED

October 1, 1985 to September 30, 1986

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Expression of Retroviral and Oncogene Proteins in Bacterial and Mammalian Vectors

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: J. A. Lautenberger Senior Staff Fellow LMO NCI

Others: F. Wong-Staal Biologist LTCB NCI
 W. G. Robey Chemist OD NCI
 N. C. Kan Senior Staff Fellow LMO NCI
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TOTAL MAN-YEARS:

1.1

PROFESSIONAL:

1.1

OTHER:

0.0

CHECK APPROPRIATE BOX(ES)

- (a) Human subjects (b) Human tissues (c) Neither
 (a1) Minors
 (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Comparison of the deduced amino acid sequences of three viral myc sequences (from avian acute leukemia viruses MC29, MH2, and OK10) with cellular proto-myc sequences from trout, chicken, mouse, and human cells indicates that there is a single position where each of the viral sequences differs from the cellular sequences. This position and its neighbors are completely conserved in the vertebrate sequences examined. Since this is unlikely to have occurred by chance alone, the mutations in this position may influence the oncogenic potential of the viruses. A simultaneous comparison of the trout, chicken, and human cellular myc sequences indicated that while on the whole these sequences are highly conserved, each sequence contains unique regions that likely were introduced by insertion events.

The genome of the AIDS virus, HTLV-III, contains an open reading frame termed sor that is conserved among different isolates of the virus. Antibodies raised against a bacterially-expressed protein containing sor sequences precipitated an Mr 23,000 protein. This protein comigrated with a protein precipitated by some sera of HTLV-III-infected humans and, thus, is likely the product of the viral sor region. When radiolabeled HTLV-III-infected T-cells were fractionated and the fractions assayed for sor by radioimmunoprecipitation, the sor protein was determined to be predominantly located in cytoplasm.

PROJECT DESCRIPTIONNames, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

J. A. Lautenberger	Senior Staff Fellow	LMO	NCI
F. Wong-Staal	Biologist	LTCB	NCI
W. G. Robey	Chemist	OC	NCI
N. C. Kan	Senior Staff Fellow	LMO	NCI
T. S. Papas	Chief	LMO	NCI

Objectives:

Important goals of the analysis of myc sequences are to determine the function of the myc proto-oncogene and to identify those features that set it apart from the myc genes of avian acute leukemia viruses. The role of mutations that produce differences between cellular and viral myc genes can be analyzed by site-directed mutagenesis of these sequences followed by a bioassay such as transfection. Additionally, the mutated sequences may be introduced into a virus and the oncogenic properties of the new virus may be compared with those of the wild-type virus.

A major aim of research concerning the AIDS virus, HTLV-III, is to determine the means by which the virus replicates and lyses the cell. A unique feature of this virus is the presence of several proteins, in addition to the gag, pol, and env proteins found in all replication competent retroviruses. Identification and characterization of these proteins should give additional insight into the host cell specificity and lytic cycle of the virus. Proteins essential for viral replication may provide attractive targets for antiviral agents.

Methods Employed:

1. Preparation of antibodies against the HTLV-III sor protein. Escherichia coli MZ1 cells containing the plasmid pSOR2 (HTLV-III sor region [nucleotides 4724-5201] in the expression plasmid pJL6) were induced at 42°C for one hour and proteins were extracted and fractionated as described by Samuel *et al.* (Gene Anal. Tech. 2: 60-66, 1985). The induced bacterial sor protein was found in the potassium thiocyanate fraction. It was solubilized in 50 mM Tris, pH 7.5, and 7 M guanidine hydrochloride and dialyzed against 5 M guanidine hydrochloride. After centrifugation the guanidine hydrochloride extract was brought to a concentration of 0.1% in trifluoroacetic acid. This protein solution was applied to a reverse phase Waters C18 μ Bonda Pack HPLC column equilibrated with 0.1% trifluoroacetic acid in water. The sor protein was eluted with a linear gradient of 0-80% of acetonitrile. A rabbit was immunized with 100 μ g of the HPLC-purified bacterial sor protein. The initial immunization was followed by two 100 μ g booster doses 10 and 20 days later. Western blot analysis performed as described (Science 226: 1094-1097, 1984) demonstrated that sera taken from the immunized rabbits contained antibodies that recognized the bacterial sor protein.

2. Detection of HTLV-III sor protein in extracts of radiolabeled cells infected with HTLV-III. Culture fluid (3 ml) containing 3×10^7 HTLV-III-infected H9 cells was labeled with 0.3 mCi of [35 S]-methionine for 18 hr. Cells were pelleted and washed two times with PBS and resuspended in lysis buffer (20 mM Hepes/5 mM KCl/5 mM MgCl₂/1% aprotinin/0.5% NP40) by repeated pipetting. Nuclei were sedimented at 3,000 x g for 10 min and washed with the same buffer. Washed nuclei were resuspended in lysis buffer containing 0.35 M NaCl and pelleted at 10,000 rpm in a SS34 rotor. The pellet was washed in lysis buffer containing 0.6 M NaCl and centrifuged for 16 hr at 40,000 rpm in a 50 Ti rotor. The nuclear pellet was resuspended in SDS-PAGE sample buffer (400 mM Tris, pH 6.8, 8% LiCl, 30% glycerol/400 mM 2-mercaptoethanol/0.02% bromophenol blue). The initial supernatant fluid was sedimented for 60 min in a Ti50 rotor yielding a "cleared cytoplasmic" supernatant and a pellet containing membranes. The membranous pellet was resuspended in SDS-PAGE sample buffer. Each fraction was subjected to immunoprecipitation by the method of R. Fisher using rabbit antibodies raised against the bacterial sor protein and human sera from HTLV-III-infected individuals. Immunoprecipitated proteins were resolved on 10% SDS-PAGE and visualized by autoradiography.

3. Preparation of extracts containing proteins that bind HTLV-I LTR sequences. The cells used were from the HTLV-I-infected T-cell line C10/MJ and the uninfected T-cell line H9. Approximately 1.5×10^8 cells were lysed by repeated pipetting with 0.1% NP40/0.34 M sucrose/10 mM NaHepes, pH 7.5/10 mM NaCl/3 mM MgCl/1 mM DTT/1 mM PMSF/0.1 mM EGTA/3 µg/ml aprotinin. Crude nuclei were sedimented at 3,000 x g for 5 min and washed with the same buffer containing 0.05% NP40 and 1 mM MgCl₂. After repeated sedimentation, nuclei were washed twice more with the same buffer containing no NP40, 0.1 mM PMSF and 0.1 mM DTT. Washed nuclei were resuspended in a volume of 5 ml of the final wash buffer containing 0.35 M KCl for salt extraction, and pipetted gently for 30 min. Salt extracted nuclei were sedimented 15 min at 10,000 x g, and the supernatant was used in DNA binding.

4. Non-denaturing polyacrylamide gel DNA binding assays. Varying amounts of proteins extracted from C10/MJ or H9 nuclei were mixed with 32 P-end labeled HTLV-I LTR restriction fragments in DNA binding buffer. The DNA binding buffer contained 10 mM Hepes, pH 7.5/50 mM KCl/1 mM EDTA/0.1 mM DTT/0.1 mM PMSF/0.1% Triton X-100/5% glycerol. Unlabeled *E. coli* DNA was included at 40 µg/ml as a non-sequence-specific competitor. Samples were loaded on 4.5% polyacrylamide gels (with an acrylamide/N,N'-methylene bisacrylamide ratio of 30/1) and electrophoresed four hours at 200 V at 4°C with a gel buffer of 10 mM Hepes, pH 7.5, 1 mM EDTA, 0.5 mM EGTA. Gels were dried and radioactivity was visualized by autoradiography.

5. Computer analysis of DNA sequences. The design of the recombinant expression plasmids made extensive use of the sequences of the DNAs involved. This analysis was greatly facilitated by the use of our Laboratory's MINC 11/23 computer and the NIH mainframe computers using software developed by ourselves and others. Programs we have written for our computer include (a) an editor to create and modify files that contain DNA sequences, (b) a program to search for restriction sites or consensus regulatory sequences, (c) a program to translate DNA sequences into amino acid sequences, (d) a program to create dot matrix homology plots of the type described by Maizel and Lenk (Proc. Natl.

Acad. Sci. USA 78: 7665-7669, 1981), (e) a program to plot termination codons and potential initiation codons in DNA sequences, (f) a program that charts the Hopp and Wood (Proc. Natl. Acad. Sci. USA 78: 3824-3828, 1981) hydrophilicity values of deduced amino acid sequences, and (g) a program to identify domains of uncharged hydrophobic amino acids that can penetrate membranes as defined by the criteria of Segrest and Feldmann (J. Mol. Biol. 87: 853-858, 1974). Our system induces a voice synthesizer that allows an investigator to proofread sequences without requiring assistance.

Simultaneous comparison of the trout deduced c-myc amino acid sequence (R. Van Beneden) with the chicken and human c-myc sequences was performed by the program of Murata *et al.* (Proc. Natl. Acad. Sci. USA 82: 3073-3077, 1985), using the MacLachlan matrix (J. Mol. Biol. 61: 409-424, 1971) for weighing pairwise amino acid comparisons. Programs were developed to display the alignments produced by this program in a more readable format.

Major Findings:

1. Detection of a unique amino acid position where viral myc oncogenes differ in sequence from cellular proto-myc genes. The DNA sequences of three avian acute transforming retroviruses that contain the v-myc oncogene, as well as the sequence of the chicken proto-myc gene, have recently been reported. We have now noticed that there is a codon where, as a result of adjacent mutations, none of the three viral sequences codes for the amino acid residue that is coded for by the cellular proto-myc gene. This is position 61 numbered from the first ATG in chicken exon 2. Position 61 and its neighbors are conserved among proto-myc genes from chicken, human, mouse and fish.

Due to the relatively low number of amino acid differences between the viral myc and chicken proto-myc sequences, it is rather unlikely that this has occurred by chance alone. Counting from the first ATG in exon 2, the chicken proto-myc locus codes for 416 amino acid residues. The number of amino acid substitutions between viral myc and chicken proto-myc is 7, 27 and 2 for MC29, MH2 and OK10, respectively. There would be only one chance in 458 that all three viral sequences contain an amino acid substitution at a common position if the substitutions occurred randomly. Substitutions at position 61 might lead to an increase in the oncogenic potential of the virus and, thus, would give the transformed cells a growth advantage.

This hypothesis is currently being evaluated by altering v-myc and animal proto-myc genes by site-specific mutagenesis to determine if substitutions at position 61 indeed modulate oncogenic potential.

2. Analysis of proteins that bind the human T-cell lymphotropic virus, Type I (HTLV-I), LTR. The long terminal repeats (LTRs) of RNA tumor viruses contain the control elements for expression of viral genes. Sequence-specific, infection-specific LTR DNA binding proteins could regulate HTLV-I functions. An *in vitro* non-denaturing polyacrylamide gel assay with restriction fragments of the HTLV-I LTR and nuclear protein extracts from the HTLV-I-infected cell line, C10/MJ, and an uninfected T-cell line, H9, was used to search for such proteins.

Nonspecific DNA binding activity was detected on all LTR restriction fragments when sufficient nuclear protein extract was used. A DNA binding activity, which is both dependent on the DNA fragment used and on the source of the extract, was also detected. A *Hinf*I restriction fragment from nucleotide +184 to +334 relative to the transcription start site produces a unique, lower mobility band on the polyacrylamide gel by binding protein from C10/MJ nuclei. Little or none of this activity was detected with an extract from H9 cells. The sequence-specific DNA binding activity was separated from other DNA binding activities by ion exchange chromatography. Protection studies map the binding site to two 10-20 bp blocks surrounding the polyadenylation site at +221.

3. Determination that the HTLV-III *sor* protein is cytoplasmic. The nucleotide sequence of the genome of HTLV-III, the infectious agent etiologically associated with Acquired Immune Deficiency Syndrome (AIDS), predicts a small open reading frame, termed *sor*, located between the *pol* and *env* genes. A DNA segment containing 82% of the *sor* region was inserted into a prokaryotic expression vector. Bacteria containing this plasmid synthesized an Mr 20,000 protein containing *sor* sequences. Antibodies to the purified bacterially-synthesized *sor* protein were found to react specifically with the same protein and also with a protein of Mr 23,000 in HTLV-III-infected H9 cells. Three serum samples from patients infected with HTLV-III were found to react with a protein that comigrated with the Mr 23,000 protein that reacted with the serum raised against the bacterial protein. The protein precipitated by these sera likely is the product of the viral *sor* gene. In order to determine the cellular location of the *sor* protein, HTLV-III-infected H9 cells were fractionated into cytoplasmic, membrane, and nuclear fractions. The Mr 23,000 protein detected by patient antisera was found to be present only in the cytoplasmic fraction.

4. Simultaneous comparison of cellular *myc* sequences. Cellular *myc* sequences from chicken, human, and trout (*Proc. Natl. Acad. Sci. USA* 83: 3698-3702, 1986) were aligned by the program of Murata et al. (see Methods). The overall alignment of these three *myc* sequences contained triple matches at 55% of the total number of positions compared. The pairwise comparisons between the sequences showed homology ranging from 62% matched residues (trout vs. human) to 68% matched residues (chicken vs. human). Smaller regions, however, contained a much higher degree of homology. Each of the sequences contained short unique regions that likely were introduced by insertion events.

Publications:

Kan, N. C., Franchini, G., Wong-Staal, F., DuBois, G. C., Robey, W. G., Lautenberger, J. A. and Papas, T. S.: Identification of HTLV-III/LAV *sor* gene product and detection of antibodies in human sera. *Science* 231: 1553-1555, 1986.

Papas, T. S., Kan, N. C., Watson, D. K., Flordellis, C., Lautenberger, J. A., Psallidopoulos, M. C., Rovigatti, U. G., Samuel, K. P., Ascione, R. and Duesberg, P. H.: Two oncogenes in avian carcinoma virus MH2: *myc* and *mht*. *Anticancer Res.* 5: 73-80, 1985.

Papas, T. S., Kan, N. C., Watson, D. K., Lautenberger, J. A., Flordellis, C., Samuel, K. P., Rovigatti, U. G., Psallidopoulos, M. C., Ascione, R. and Duesberg, P. H.: Myc, a genetic element that is shared by a cellular gene (proto-myc) and by viruses with one (MC29) or two (MH2) onc genes. In Furmanski, P., Hager, J.-C. and Rich, M. A. (Eds.): RNA Tumor Viruses, Oncogenes, Human Cancer and AIDS: On the Frontiers of Understanding. Boston, Martinus Nijhoff Publishing, 1985, pp. 1-14.

Papas, T. S., Kan, N. C., Watson, D. K., Lautenberger, J. A., Flordellis, C., Samuel, K. P., Rovigatti, U. G., Psallidopoulos, M., Ascione, R. and Duesberg, P. H.: Oncogenes of avian acute leukemia viruses are subsets of normal cellular genes. In Neth, R., Gallo, R. C., Greaves, M. F. and Janka, G. (Eds.): Haematology and Blood Transfusion, Modern Trends in Human Leukemia VI. Berlin/Heidelberg, Springer-Verlag, 1985, Vol. 29, pp. 269-272.

Papas, T. S. and Lautenberger, J. A.: Sequence curiosity in v-myc oncogenes. Nature 318: 237, 1985.

Papas, T. S., Lautenberger, J. A., Watson, D. K., Fisher, R. J., Fujiwara, S., Kan, N. C., Samuel, K. P., Flordellis, C., Psallidopoulos, M., Zhou, R. P., Seth, A., Duesberg, P. and Ascione, R.: Viral myc genes and their cellular homologs. In Papas, T. S. and Vande Woude, G. F. (Eds.): Gene Amplification and Analysis: Oncogenes. New York, Elsevier, Vol. 4 (In Press)

Papas, T. S., Samuel, K. P., Kan, N. C., Ascione, R., Wong-Staal, F. and Lautenberger, J. A.: Production of oncogene-specific proteins and human T-cell leukemia (lymphotropic) retrovirus (HTLV-I) envelope protein in bacteria and its potential for use in human malignancies and seroepidemiological surveys. Cancer Res. 45: 4568s-4573s, 1985.

Shih, T. Y., Hattori, S., Clanton, D. J., Ulsh, L. S., Chen, Z., Lautenberger, J. A. and Papas, T. S.: Structure and function of p21 ras proteins. In Papas, T. S. and Vande Woude, G. F. (Eds.): Gene Amplification and Analysis: Oncogenes. New York, Elsevier, Vol. 4 (In Press)

Sisk, W. P., Chirikjian, J. G., Lautenberger, J., Jorcyk, C., Papas, T. S., Berman, M., Zagursky, R. and Court, D. L.: A vector for selection and expression of gene segments: Expression of an HTLV-I envelope gene segment. Gene (In Press)

Van Beneden, R. J., Watson, D. K., Chen, T. T., Lautenberger, J. A. and Papas, T. S.: Cellular myc (c-myc) in fish (rainbow trout): Its relationship to other vertebrate myc genes and to the transforming genes of the MC29 family of viruses. Proc. Natl. Acad. Sci. USA 83: 3698-3702, 1986.

Patents:

None

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01CP05238-05 LMO

PERIOD COVERED

October 1, 1985 to September 30, 1986

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

The Transforming Genes of Acute Leukemia Viruses and Their Cellular Homologues

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	D. K. Watson	Senior Staff Fellow	LMO	NCI
Others:	T. S. Papas	Chief	LMO	NCI
	S. J. O'Brien	Chief	LVC	NCI
	N. Sacchi	Visiting Associate	LMO	NCI
	R. J. Fisher	Expert	LMO	NCI
	N. K. Bhat	Visiting Fellow	LMO	NCI
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	L. J. Pribyl	Biologist	LMO	NCI

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1.9

PROFESSIONAL:

0.9

OTHER:

1.0

CHECK APPROPRIATE BOX(ES)

- (a) Human subjects (b) Human tissues (c) Neither
- (a1) Minors
- (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

To provide an initial step toward the understanding of the functional relationship between the onc genes of transforming retroviruses and their cellular prototypes, structural comparisons at the nucleic acid and protein levels have been carried out. We have determined the complete nucleotide sequence of the chicken ets gene and compared it to the ets gene of E26. E26 is a genetic hybrid with sequences derived from viral structural genes and parts of essential cellular proto-onc genes. The cellular genes contain additional 5' sequences. The substitution of viral genes for parts of the normal cellular genes may be the most significant difference between these genes, perhaps eliciting functional differences between their gene products. The avian cellular gene transcript is considerably larger than the amount of DNA transduced by the virus. We have determined that the mammalian homologues of v-ets consist of two distinct domains located on different chromosomes. Using chromosome-specific probes, we have shown that both loci (ets-1 and ets-2) are transcriptionally active, producing mRNA species. The sequences homologous to ets-1 and ets-2 are colinear in chicken proto-ets and have possibly become separate and functionally distinct since before the evolutionary divergence of Mammalia. The mammalian ets genes from man and mouse encode for essentially identical amino acids, and are over 90% conserved relative to the chicken ets gene. We have utilized synthetic peptides and bacterially-expressed proteins to elicit production of antibodies which specifically immunoprecipitate ets protein. DNA from human cell lines and tumors has been analyzed for polymorphism and/or gene rearrangement. Both ets-1 and ets-2 genes translocate from their normal position in cells presenting 11q23 and 22q22 rearrangements.

PROJECT DESCRIPTIONNames, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

D. K. Watson	Senior Staff Fellow	LMO	NCI
T. S. Papas	Chief	LMO	NCI
S. J. O'Brien	Chief	LVC	NCI
N. Sacchi	Visiting Associate	LMO	NCI
R. J. Fisher	Expert	LMO	NCI
N. K. Bhat	Visiting Fellow	LMO	NCI
S. Fujiwara	Visiting Fellow	LMO	NCI
L. J. Pribyl	Biologist	LMO	NCI

Objectives:

The purpose of this investigation is to determine the relationship between onc genes and their normal cellular homologues. Structural analysis of these genes will allow us to better understand their biological functions. In addition, we plan to evaluate the involvement of proto-onc genes in human malignancy.

Methods Employed:

1. Preparation of high molecular weight DNA from eukaryotic cells by treatment with proteinase K, followed by phenol-chloroform extraction. Vector DNA was prepared from phage and plasmid derivatives by phenol extraction and CsCl gradient centrifugation.
2. Digestion of DNA with restriction enzymes and resolution of the resultant fragments on agarose and/or polyacrylamide gels. Specific DNA fragments were purified by electroelution or by extraction of low melting agarose.
3. Preparation of DNA probes using purified onc-specific DNA by nick-translation using E. coli DNA polymerase and DNase I.
4. Preparation of nitrocellulose filters containing immobilized restriction fragments by the Southern blot technique and hybridization of onc probes.
5. Construction of recombinant phage libraries by ligation of restriction fragments of eukaryotic DNA to cosmid or λ vector DNA followed by production of phage by in vitro packaging.
6. Isolation of phage from the libraries containing virus-related sequences by hybridization of onc-specific probes to nitrocellulose filters containing phage DNA prepared from plaques lifted from petri plates by the procedure of Benton and Davis (Science 196: 180-182, 1977). Isolation of plasmid DNA from colonies lifted from plates by the method of Grunstein and Hogness (Proc. Natl. Acad. Sci. USA 72: 3961-3965, 1975).
7. Subcloning of isolated DNA fragments into pBR322, pBR325, or pBR328, as required.

8. DNA sequencing analysis of cloned DNA by the method of Maxam and Gilbert (Methods Enzymol. 65: 499-560, 1980). In addition, uniquely-labeled DNA will be sequenced following RNA-directed primer extension.
9. Total cellular RNA from cultured cells or tissues was prepared by the guanidine hydrochloride method. In addition, RNA was prepared after cellular fractionation in the presence of RNase inhibitors. Separation of polyA+ and polyA- RNA by two cycles of purification through oligo(dT) cellulose. Characterization of RNA by electrophoresis on formaldehyde-agarose or methylmercury-agarose and Northern analysis.
10. Construction of cDNA library: Double-stranded cDNA was prepared from polyA+ RNA and ligated into λ gt10 vector DNA for amplification.
11. Immunoprecipitation of labeled cell lysates with onc-specific antisera and analysis by SDS-polyacrylamide gel electrophoresis. The specific onc gene-related polypeptide was purified for further analysis by electroelution.
12. S^{35} -methionine and -cysteine labeling of expressed protein in bacteria utilizing the plasmid, pJL6 (vector constructed by Dr. James Lautenberger).
13. Tryptic peptide analyses of viral and cellular onc-related polypeptides by two-dimensional, thin-layer electrophoresis and chromatography.
14. Microsequence analysis of onc-related polypeptides to define the site of initiation of translation.

Major Findings:

1. Sequences related to ets (one of the two cellular genes present in the avian retrovirus, E26) have been detected by restriction enzyme digestion and Southern blot analysis using genomic DNA derived from avian and mammalian species.
2. DNA prepared from panels of hamster X human and mouse X human somatic cell hybrid clones, which have lost specific human chromosomes, were used for analyses to determine the chromosomal localization of ets. The v-ets homologous sequence is dispersed as two genetic loci, ets-1 and ets-2. These loci contain sequentially distinct domains of v-ets and are mapped to chromosomes 11 and 21.
3. The assignment of ets-1 to chromosome 11 is consistent with the recent study of de Tainshe et al. (Nature 310: 581-583, 1984), who reported the assignment of ets to 11q23-24. Our in situ hybridization of an ets-2 clone to normal human chromosome preparations confirmed the assignment of ets-2 to chromosome 21 and regionally localized to HSA 21q22.1-22.3.
4. Using a previously characterized panel of mouse X hamster hybrids (J. Virol. 49: 297-299, 1984), we have been able to assign ets-1 and ets-2 to murine chromosomes 9 and 16.
5. The domestic cat homologues of the ets proto-oncogenes were found to be D1 (ets-1) and C2 (ets-2).

6. When viral probes related to ets-1 and ets-2 are hybridized to a single chicken c-ets clone, we find that sequences related to both loci are present in this clone. From overlapping chicken clones, the v-ets related exons are dispersed over 35 kb of genomic DNA.
7. Chicken, mouse and human genomic libraries were screened and specific clones have been isolated. In addition, human cDNA for ets-1 and ets-2 has been isolated. Chicken and mouse cDNA clones will be isolated and subjected to nucleotide sequence analysis.
8. Portions of the human ets-1 and ets-2 loci have been sequenced, demonstrating a strong conservation of amino acids (over 90%), suggesting that this gene performs an important function. The mouse ets-2 gene is essentially identical to that of human.
9. Both genetic loci are transcriptionally active in man, yielding distinct products.
10. The expression of ets-1 and ets-2 genes in human cell lines has been assayed by Northern blot analyses. The pattern seen suggests that these two genes are independently regulated.
11. Viral ets fragments, and human ets exons and cDNA have been expressed in bacteria. Following purification these proteins have been used to elicit antibody response.
12. Human ets-1 and ets-2 genes both translocate from their normal position as a consequence of rearrangements involving the 11q23 and 22q22 breakpoints.
13. A large coding segment of human ets-2 has been inserted into a eukaryotic expression vector under the transcriptional control of the heat shock promoter. Such constructs will be microinjected into fertilized mouse eggs to assess the role of ets-2 during development, and to establish cell lines with inducible ets-2.
14. To provide a genetic system affording many manipulations, we have isolated a Drosophila ets gene. The gene we have isolated is highly conserved at the amino acid level, with greater than 90% of the ets-2 region maintained. The expression of the ets-2 gene during development is not constant, but related to periods of differentiation.
15. Expression of ets genes in mouse tissues and during development is not uniform. Highest expression has been observed in the liver following partial hepatectomy and in developing testes.

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Patents:

None

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01CP05239-05 LMO

PERIOD COVERED

October 1, 1985 to September 30, 1986

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Structural Analysis of the Avian Carcinoma Virus MH2

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	N. C. Kan	Senior Staff Fellow	LMO	NCI
Others:	T. S. Papas	Chief	LMO	NCI
	J. A. Lautenberger	Senior Staff Fellow	LMO	NCI
	D. G. Blair	Supervisory Research Chemist	LMO	NCI
	Q. Yuan	Guest Researcher	LMO	NCI

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1.1

PROFESSIONAL:

1.1

OTHER:

0.0

CHECK APPROPRIATE BOX(ES)

- (a) Human subjects (b) Human tissues (c) Neither
- (a1) Minors
- (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Avian carcinoma virus MH2 contains two potential transforming genes, v-mht and v-myc. We have constructed deletion and frameshift mutants of each of the two genes by in vitro mutagenesis of cloned MH2 proviral DNA and tested their transforming function and virus production in cultured primary quail cells. We have found that the v-myc gene transformed primary cells by itself without the second potential oncogene. v-mht was without detectable transforming function but may affect transformation by v-myc. We are currently investigating the possibility that v-mht may have an enhancing function of v-myc in animals. To study the dual oncogenes in another avian retrovirus, E26, we have constructed a replication-defective murine retrovirus carrying the v-myb and v-ets oncogenes derived from E26. The DNA construct, termed ME26, was transfected into NIH3T3 cells, together with pSV2neo, and G418-resistant colonies were selected. These cells were infected with a murine helper virus, and Northern analysis using a v-ets-specific probe indicated that the recombinant ME26 virus was present in the supernatant. The rescued virus could be successfully passed to NIH3T3 cells by infection.

We have inserted a DNA segment containing 82% of the sor open reading frame of HTLV-III/LAV virus into a prokaryotic expression vector, pJL6. The bacterially-synthesized sor protein reacted with sera from individuals infected with HTLV-III, indicating that sor was expressed as a protein product that was immunogenic in vivo. Antibodies to the purified, bacterially-synthesized sor protein immunoprecipitated a 23-kilodalton protein in HTLV-III-infected H9 cells, suggesting that this protein may be the sor gene product.

PROJECT DESCRIPTIONNames, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

N. C. Kan	Senior Staff Fellow	LMO	NCI
T. S. Papas	Chief	LMO	NCI
J. A. Lautenberger	Senior Staff Fellow	LMO	NCI
D. G. Blair	Supervisory Research Chemist	LMO	NCI
Q. Yuan	Guest Researcher	LMO	NCI

Objectives:

To study the interaction and functional relationship between dual oncogenes captured by retroviruses using avian acute leukemia viruses MH2 and E26 as model systems. In particular, the following lines of investigation were addressed: (1) examination of the oncogenicity of v-mht either by itself or in concert with v-myc in MH2; (2) construction of a replication-defective murine retrovirus carrying the v-myb and v-ets oncogenes derived from E26, and (3) examination of the oncogenicity of v-myb and v-ets and their interaction in NIH3T3 cells and in nude mice. To characterize the gene product of the sor open reading frame of HTLV-III/LAV virus; to study the immunogenicity of the sor protein in AIDS patients; to determine the subcellular localization of the sor protein in HTLV-III/LAV-infected cells and to elucidate its function.

Methods Employed:

1. Construction of a murine retrovirus and deletion mutants: (a) digestion of recombinant plasmid DNA with restriction enzymes, (b) preparation of blunt-ended DNA fragments by repairing the ends with E. coli DNA polymerase large fragments, (c) elimination of restriction enzyme sites using restriction enzyme-associated methylases, (d) addition of restriction enzyme linkers to DNA fragments by blunt-end ligation using T4 DNA ligase, (e) cohesive-end ligation using T4 DNA ligase, (f) use of small preparations of recombinant DNA (minilysates) to identify the desired DNA clones, (g) colony hybridization using nick-translated radioactive DNA probes to screen for desired recombinant DNA clones, (h) gel electrophoresis analysis of recombinant DNA fragments on agarose or polyacrylamide gels, and (i) DNA sequence analysis by the chain terminator method of Sanger et al. (Proc. Natl. Acad. Sci. USA 74: 5463-5467, 1977) modified by Zagursky et al. (Gene Anal. Tech. 2: 89-94, 1985) to determine the precise nature of the DNA constructs.
2. Cloning of DNA fragments into M13-derived vectors mp8, mp18, and mp19 and DNA sequencing using the chain terminator method.
3. Insertion of the HTLV-III/LAV sor open reading frame into the expression vector, pJL6, using the methods described in (1) and ³⁵S-methionine and -cysteine labeling of expressed protein in E. coli.

4. Preparation of nitrocellulose filters containing immobilized DNA fragments or RNA molecules by the Southern or Northern blot techniques and hybridization with radioactive DNA probes.
5. Immunoprecipitation of labeled cell extract with antisera against purified sor protein expressed in E. coli and analysis by SDS-polyacrylamide gel electrophoresis.

Major Findings:

1. Deletion and frameshift mutants of each of the two MH2 genes, v-mht and v-myc, were prepared. Studies on these mutants indicated that the v-myc gene transformed avian primary cells in vitro by itself, without the second potential oncogene. On the other hand, the v-mht gene did not show detectable transforming ability but may affect transformation of avian primary cells in cooperation with the v-myc gene.
2. A replication-defective murine retrovirus carrying the v-myb and v-ets oncogenes derived from the avian retrovirus E26 was constructed. DNA sequence analysis verified that the E26-derived insertion was in frame with the first 34 codons of murine gag p15. It should thus encode a murine gag-avian gag-myb-ets fusion protein of approximately 133 kilodaltons. The construct, termed ME26, was transfected into NIH3T3 cells, together with pSV2neo, and G418-resistant colonies were selected. Thirteen of 18 isolated colonies contained integrated ME26 sequences.
3. Recombinant ME26 virus was rescued from the supernatant of transfected NIH3T3 cells upon superinfection with a murine helper virus. The rescued ME26 virus could be successfully passed to NIH3T3 cells by infection, without gross alterations of the recombinant genome.
4. A protein of about 133 kilodaltons was detected in NIH3T3 cells transfected with the ME26 construct. This protein reacted specifically with the anti-avian gag and anti-ets antisera and may represent the fusion protein described in (2).
5. A DNA segment containing 82% of the sor open reading frame of HTLV-III/LAV was inserted into a prokaryotic expression vector, pJL6, to mass produce a partial sor protein in E. coli. The bacterially-synthesized sor protein reacted with sera from individuals infected with HTLV-III, indicating that sor was expressed as a protein product that was immunogenic in vivo.
6. Antibodies to the purified, bacterially-synthesized sor protein were found to react specifically with a protein of 23 kilodaltons in HTLV-III-infected H9 cell extracts, suggesting that this protein may be the sor gene product.
7. The 23-Kd sor protein was found in the cytoplasmic fraction of HTLV-III-infected H9 and Molt 3 cells by immunoprecipitation with the sor-specific antibodies, indicating that the sor gene product was a cytoplasmic protein.

Publications:

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Patents:

None

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01CP05288-05 LMO

PERIOD COVERED

October 1, 1985 to September 30, 1986

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Control of Developmental Gene Expression - Chromatin Structure of Active Genes

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: D. D. Blumberg Senior Staff Fellow LMO NCI

Other: J. F. Comer Microbiologist LMO NCI

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TOTAL MAN-YEARS:

1.85

PROFESSIONAL:

0.85

OTHER:

1.0

CHECK APPROPRIATE BOX(ES)

- (a) Human subjects (b) Human tissues (c) Neither
- (a1) Minors
- (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

A very simple model system, the cellular slime mold, Dictyostelium discoideum, is being used to study the mechanisms which control developmental gene activation during normal differentiation. Because of the similarity of basic regulatory mechanisms, this system represents an attractive alternative to animal research. During growth and the early stages of aggregation Dictyostelium cells express 50-55% of their single copy genome as mRNA and HnRNA. An additional 26% of the single copy genome is expressed only during the late stages of development. Initiation of transcription on the late portion of the genome requires cell-cell interaction and cAMP. Because such a high proportion of this small eukaryotic genome is either constitutively transcribed or developmentally induced, it offers a unique opportunity to study the structural organization in chromatin of transcriptionally active genes. Our results indicate that both the constitutively expressed and developmentally inducible genes are in a DNase I-sensitive, active structure in chromatin regardless of whether the developmentally inducible genes are being transcribed. By contrast, micrococcal nuclease has been used to identify a structural organization unique to genes which are actually in the process of being transcribed. Properties of this organization have been used to resolve oligonucleosomes specifically derived from actively transcribed genes and to determine their protein composition. Nucleosomes from transcriptionally active genes are devoid of histone H1, while those from inactive genes contain H1.

PROJECT DESCRIPTIONNames, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

D. D. Blumberg	Senior Staff Fellow	LMO	NCI
J. F. Comer	Microbiologist	LMO	NCI

Objectives:

The aim of these studies is to understand the mechanisms which control developmental gene activation during normal differentiation. A very simple model system, the cellular slime mold, Dictyostelium discoideum, is being employed.

The predominant feature of the developmental cycle of Dictyostelium discoideum is the aggregation of unicellular, free-living amoeba into a multicellular organism. Differentiation of amoeba within the newly formed aggregates generates the three distinct cell types found in the mature fruiting body: spore cells, stalk cells, and basal disks. Dictyostelium exhibits many features of development seen in more complex eukaryotic organisms. Specific cell-cell contacts are found, a homogeneous population differentiates into discrete cell types, and there is specific cell migration and pattern formation. These morphogenetic changes are accompanied by major changes in the pattern of gene expression. In particular, post-aggregation Dictyostelium cells contain 2000-3000 new messenger RNA species that are absent from earlier preaggregation-stage cells. These new aggregation-dependent sequences compose 30% of the mass of the messenger RNA in the late cells and, together with their heteronuclear RNA precursors, represent the transcription products of an additional 26% of the single copy portion of the genome.

The initiation of transcription in post-aggregation cells occurs on a portion of the genome, and appears to be dependent upon cell-cell interaction. Additionally, both the rate of transcription and subsequent stability of the mRNAs transcribed off of this portion of the genome are further regulated by a cAMP-mediated process.

The major objective of our work is to elucidate the molecular mechanisms by which these genes are coordinately activated, and to understand the controls which regulate the transcription rate and stability of these mRNAs in response to environmental stimuli--in this case--cell contact and cAMP. In order to achieve this objective, several closely-integrated approaches are being employed. In the first approach described in this summary, we are exploring the basic aspects of the structural organization of these genes, isolated as chromatin, with the objective of understanding the nucleoprotein interactions which are important in activation of gene expression.

Dictyostelium's chromatin offers a unique opportunity to study the structure of actively transcribed genes or genes which will become active in response to a developmental stimulus. Unlike the chromatin from higher cells, in which less than 20% of the DNA is ever transcribed, nearly 80% of the Dictyostelium genome is transcribed. Fifty percent is transcribed during growth, while an additional

30% is active during late development. Thus, analysis of bulk *Dictyostelium* chromatin is essentially an analysis of the domain of active chromatin. Additionally, because of the low complexity of the genome, one can probe the structural organization of single-copy genes in their nucleoprotein forms.

Methods Employed:

Standard molecular cloning techniques are being employed to isolate and map recombinant DNAs encoding constitutively expressed and developmentally regulated genes. Two endonucleases, DNase I and micrococcal nuclease, are being used to probe the structural organization of genes in chromatin, while a variety of agarose and polyacrylamide gel systems are being utilized, in one and two dimensions, in order to resolve nucleoprotein complexes, and in histones, as well as the DNA, and RNA species.

Major Findings:

Use of micrococcal nuclease to detect a chromatin structure specifically associated with gene transcription: The enzyme micrococcal nuclease preferentially digests chromatin at sites in the linker region between nucleosomes. In higher eukaryotes in which the bulk of the DNA (80-90%) is transcriptionally inactive, a precise repeating ladder of bands is observed when the digested DNA is resolved on agarose gels. The lengths of the bands are integral multiples the size of the smallest unit of the repeat, the mononucleosome. *Dictyostelium's* chromatin is unique in that the bulk is active, and all of the genes are in a DNase I-sensitive configuration (see above). Unlike the very precise nucleosome repeat ladder observed in higher eukaryotes, the *Dictyostelium* pattern is somewhat more diffuse and irregular. This irregular repeat pattern has been analyzed for individual genes which fall into the three transcriptional classes: (1) genes which are transcribed at a moderate rate throughout growth and development; (2) genes which are not transcribed at all in the growing cells, but are expressed only during late development; and (3) genes which are expressed at a very low level in growing cells and induced to a higher level of transcription at the time when cell contact is formed. The differentiation-specific genes, which are transcriptionally inactive in the growing cells from which the chromatin was prepared, give a very precise repeat ladder with a unit length of 175 nucleotides and resemble the patterns seen for bulk inactive chromatin in higher cells. By contrast, the genes which are transcribed at a very low level in the growing cells show a ladder of bands which are not integral multiples of the 175 base pair repeat. Instead, they appear as an irregular ladder of bands with a spacing of about 80 nucleotides between bands. The genes which are transcribed at a higher rate in the growing cells show the same nonintegral repeat as the genes expressed only at a very low level. However, the irregular ladder of bands is now superimposed on a background smear which is similar to the smear seen in higher eukaryotes when probes for very actively transcribed genes are hybridized to micrococcal nuclease digests of chromatin. This irregular ladder of bands that we detect in the transcribed genes probably represents a structural intermediate between the very regularly spaced ladder seen upon micrococcal nuclease digestion of inactive chromatin and the smear seen in association with genes transcribed at a very high rate. This irregular ladder is probably detected because the genes whose structures are being probed are transcribed at a very low rate. Analysis of the location of these cuts

should enable us to identify sites on nucleosomes associated with the actively-transcribed genes which are more accessible and open to cutting by micrococcal nuclease. At present this irregular ladder of bands is consistent with at least one of two different interpretations. If the irregular ladder of bands results from the actively-transcribed genes being cut predominantly at sites which are approximately 80 base pairs apart, as opposed to the 175 base pairs seen for the inactive genes, then transcription of the genes is resulting in exposure of specific sites within the nucleosome core, as well as at the usual site of micrococcal nuclease attack in the linker region. At the very low levels of transcription, a single or limited number of sites on the core particle may be accessible. At higher levels of transcription the nucleosome core may become progressively more open, resulting in many additional exposed sites accessible to micrococcal nuclease digestion and giving rise, ultimately, to the smear. The second interpretation is that the irregular pattern of bands arises as a result of interspersed closely-packed core particles lacking a linker region and histone H₁ with normal nucleosomes containing histone H₁ and the linker region. Thus, the possibility that the irregular length DNA bands arise from closely-packed nucleosome cores cannot be discounted. A model in which one has interspersed closely-packed core particles and core particles with linker regions, possibly resulting from a reduction in the amount of histone H₁ associated with these genes, is entirely consistent with the size of DNA bands that we see.

Two-dimensional hybridization mapping of dinucleosomes indicates histone H₁ is lacking from transcriptionally active genes: Soluble mono- and oligonucleosomes have been isolated from nuclei from growing cells following micrococcal nuclease digestion. These mono- and oligonucleosomes have been resolved by low ionic strength polyacrylamide gel electrophoresis. In addition to the two mononucleosome species corresponding to the nucleosome core particle MN₁ (140 bp DNA and the octamer of histones), and to the core particle plus histone H₁, MN₂, there are four other deoxynucleoprotein particles termed A, B, C and D that migrate in the region that normally contains the dinucleosomes. The DNA associated with these particles was electrophoresed into a 2^d DNA gel, electrotransferred to gene screen, UV cross-linked, and hybridized to the transcriptionally-active and -inactive cDNA probes. DNA from particles A and B hybridizes to the cDNA probe for the developmentally-regulated, transcriptionally-inactive genes and is the appropriate length (315-330 nucleotides) for a regular dinucleosome. Particles C and D are associated with DNA in the range of 240 bases up to 315 bases--a size range shorter than dinucleosome length and characteristic of the nonintegral DNA length associated with active genes. Indeed, this DNA hybridizes almost exclusively with the probes for transcriptionally-active genes and shows very little hybridization with the probes for inactive genes. When the proteins associated with nucleoprotein particles A, B, C and D are analyzed by electrophoresis in a 2^d acid urea protein gel, histone H₁ is clearly present in particles A and B, and clearly absent from particles C and D. Particles C and D, however, are associated with a full complement of the core histones, H₂A, H₂B, H₃ and H₄. It would appear that active genes in growing Dictyostelium cells are present in nucleosomes which have lost histone H₁ and which contain nonintegral length DNA fragments.

Publications:

Oyama, M. and Blumberg, D. D.: Changes during differentiation in requirements for cAMP for expression of cell-type specific mRNAs in the cellular slime mold, Dictyostelium discoideum. Dev. Biol. (In Press)

Oyama, M. and Blumberg, D. D.: Cyclic AMP and $\text{NH}_3/\text{NH}_4^+$ both regulate cell-type specific mRNA accumulation in the cellular slime mold, Dictyostelium discoideum. Dev. Biol. (In Press)

Oyama, M. and Blumberg, D. D.: Interaction of cAMP with the cell surface receptor induces cell-type specific mRNA accumulation in Dictyostelium discoideum. Proc. Natl. Acad. Sci. USA (In Press)

Patents:

None

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01CP05295-05 LMO

PERIOD COVERED

October 1, 1985 to September 30, 1986

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Studies on the Activation of onc Genes in Viruses and Human Tumors

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	D. G. Blair	Supv. Research Chemist	LMO	NCI
Others:	T. S. Papas	Chief	LMO	NCI
	N. C. Kan	Senior Staff Fellow	LMO	NCI
	K. J. Dunn	Bio. Lab. Tech. (Micro.)	LMO	NCI
	Q. Yuan	Guest Researcher	LMO	NCI

COOPERATING UNITS (if any) Mol. Mech. of Car. Lab., Basic Research Program, LBI, Frederick, MD (G. F. Vande Woude, A. Seth, M. Park, M. Schmidt, T. Robins, M. K. Oskarsson); Nucl. Acid & Protein Syn. Lab., PRI, Frederick, MD (M. Zweig, S. D. Showalter, L. A. Eader); Lab. of Cell & Mol. Structure, PRI, Frederick, MD (A. Boyd)

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Laboratory of Molecular Oncology

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2.75

PROFESSIONAL:

1.75

OTHER:

1.0

CHECK APPROPRIATE BOX(ES)

- (a) Human subjects (b) Human tissues (c) Neither
- (a1) Minors
- (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

We have detected two transfectable transforming sequences following the screening of DNA from a human ovarian carcinoma cell line, OVCAR3, in the nude mouse tumor assay. The morphologically distinguishable transforming activities show differential response to the glucocorticoid dexamethasone (DEX) which abolishes our ability to detect morphological transformation induced by one of the two sequences. We have cloned a conserved *alu* + 10.5-kb fragment from this DEX-sensitive transformant. It represents the fusion of two normally unlinked sequences which apparently occurred during transfection.

We have constructed a murine retrovirus (ME26) which carries avian *gag*, as well as *v-myb* and *v-ets* sequences derived from the avian erythroleukemia virus, E26. The virus replicates to high titer when rescued by replicating murine leukemia virus, and cells infected with the virus express a 140-Kd fusion protein which reacts with anti-avian *gag* and anti-*ets* antisera. The virus does not appear to transform NIH3T3 mouse embryo fibroblasts.

We have transfected a bovine papillomavirus (BPV)-based construct which contains the human metallothionein gene and the murine sarcoma virus *mos* gene, either fused to human growth hormone (HGH) sequences under metallothionein control, or fused directly to a metallothionein promoter. Both constructs appear to transform mouse C127 cells and NIH3T3 cells. Very high levels of *mos* fusion protein can be detected in the presence of cadmium only in cells transformed by the HGH fusion construct.

PROJECT DESCRIPTIONNames, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

D. G. Blair	Supv. Research Chemist	LMO	NCI
T. S. Papas	Chief	LMO	NCI
N. C. Kan	Senior Staff Fellow	LMO	NCI
K. J. Dunn	Bio. Lab. Tech. (Micro.)	LMO	NCI
Q. Yuan	Guest Researcher	LMO	NCI

Objectives:

To understand the mechanism of transformation by murine sarcoma viruses (MSV) and the function of specific gene products of MSV in this process.

To define the functions of specific portions of the Moloney MSV genome in MSV transformation and to identify specific genetic sequences necessary to activate the transformation potential of normal cell sequences of mouse and human origin.

To study the mechanism and cooperative role of the myb and ets oncogenes in the avian erythroleukemia virus, E26, and to evaluate their transforming potential in other species.

To develop screening and selection systems to identify and isolate human transforming DNA sequences from primary human tumors and tumor cell line DNAs. To identify, isolate and characterize such sequences and their gene products. To characterize the normal cellular homologs of such sequences and to determine the mechanism by which their oncogenic potential is activated.

Methods Employed:

Standard methods of tissue culture of transformed and normal cells, DNA and RNA isolation from tumor tissues and tissue culture, Southern and Northern blotting using radioactive probes prepared from cloned DNAs, calcium-phosphate-DNA transfection, measurement of tumorigenicity of cell lines in nude and other strains of mice, immunoprecipitation and protein gel analysis to detect antibodies to specific cellular proteins.

Major Findings:

1. One of two transfectable transforming sequences identified after transfection of human ovarian carcinoma cell DNA was activated during transfection. We have previously reported that transfection of DNA from the human ovarian carcinoma-derived cell line OVCAR3 into NIH3T3 cells resulted in the generation of tumors following the injection of transfected cells into nude mice. Three out of four primary tumors induced 2° tumors upon transfection of their DNA, and foci of morphologically-transformed cells were also detected. None of the tumors or foci contain human ras-related sequences. Two out of three positive tumors induced only compact dense foci, while the third induced both

this type of focus, as well as a more diffuse refractile focus. In the presence of dexamethasone (0.125 μM), only the refractile foci could be detected, suggesting a hormone-sensitive function or control element associated with the dense focus transformation. The two types of 2° foci exhibited different patterns of human repeat (alu) hybridization, supporting the hypothesis that they represented different transforming sequences. A single 10.5-kb R1 fragment was cloned from a dense morphology 2° focus and two alu-free subclones were isolated which were separated by approximately 7 kb of alu-containing human sequences. Hybridization of the alu-free probes to R1-digested OVCAR3, as well as other human normal and tumor DNAs, revealed that each hybridized to a single unique sized fragment, but did not hybridize to mouse DNA. This indicates that at least some of the transfected human sequences were generated by the fusion of two unlinked DNA fragments, probably during the transfection, and raises the possibility that the transforming sequence we detect was also activated as a result of this fusion.

2. A rescuable murine retrovirus carrying the myb and ets oncogene sequences of the avian erythro leukemia virus, E26, has been generated. The acute avian erythro leukemia virus, E26, which has acquired two cellular sequences, v-myb and v-ets, induces primarily erythroblastosis with some myeloblastosis in vivo, and transforms erythroblasts, myeloblasts and quail fibroblasts in vitro. To study the oncogenic potential of these well-conserved genes in other species, as well as to investigate the nature of any cooperative interaction between them, we have constructed a retrovirus carrying these sequences and capable of replication in mouse cells. The DNA fragment containing a portion of the avian gag sequences and the entire v-myb and v-ets sequences from E26 were inserted in frame and fused to murine p15 gag sequences in a mouse leukemia virus (MuLV)-derived vector. The construct, termed ME26, expressed a ~140,000-dalton protein when transfected into NIH3T3 mouse cells, which reacted with avian gag-specific and ets peptide-specific antisera. The virus could be rescued effectively with Moloney MuLV and could be successfully transmitted to fresh NIH3T3 cells and mouse embryo fibroblasts by infection. Infected cells produced essentially equal levels of MuLV and ME26 viruses, but no evidence of morphological transformation could be detected. The biological activity of the viruses in mouse hematopoietic cells in vitro and in mice in vivo is currently being evaluated.

3. Infection by a retroviral construct containing the gene for hygromycin phosphorylase is capable of conveying hygromycin resistance. Many, if not all, mammalian cells are sensitive to the aminoglycosidic antibiotic hygromycin B, and upon acquiring the gene for hygromycin B phosphorylase are rendered resistant. The hygromycin-resistance gene was substituted for the G418-resistance gene in pSV₂neo, resulting in a new construct, pSV₂hygro. The hygromycin-resistance gene was also substituted for the G418-resistance gene in a retroviral vector. The constructs were tested for biologic activity in mouse, human, and cat fibroblasts by transfection and drug selection. Hygromycin resistance was conveyed via transfection with an efficiency comparable to that achieved with G418, and it was demonstrated that the two drug-resistance traits could be independently selected. Cotransfectants could also be selected for resistance to hygromycin and G418 simultaneously. Mixed viral stocks were generated from fpHV-1 transfectants with a pseudotype titer for conveying drug-resistance of 10⁵ colony-forming units per ml. Southern analysis of genomic DNA from infected and transfected cells showed a single copy of fpHV-1

in infectants and multiple copies in transfectants. It is expected that retroviral vector infection using hygromycin and/or G418 selection will be suitable for the stable cotransfer of selected cloned genes.

4. A v-mos human growth hormone (HGH) fusion protein under metallothionein promoter control transforms mouse cells and is expressed at high levels in the presence of cadmium. We transfected C127 cells with a BPV-based vector containing v-mos sequences either fused to HGH and under metallothionein control, or linked directly to a metallothionein promoter. Morphological transformation was detected in the absence of cadmium (Cd), but was much more pronounced in cells selected for Cd resistance. Resistant cells contained high levels of unintegrated mos-containing DNA (>20 copies) and a low number of apparently integrated copies. In the presence of Cd, high levels of mos fusion protein were detected in cells transformed by the HGH-mos construct by either Western blot, immunoprecipitation or immunofluorescent analysis using an anti-mos-specific serum. No mos protein was detected in cells transfected with the unfused mos construct. The analysis of purified mos protein produced in mammalian cells is in progress.

5. Analysis of a somatic cell hybrid containing chromosomes marked with retroviruses carrying drug resistance markers demonstrates that the met oncogene was translocated before activation. Analysis of human-rodent somatic cell hybrids is complicated by the frequent and variable loss of human chromosomes from such hybrids in the absence of a selectable marker. In an attempt to devise a method to apply drug selection techniques to maintain any desired human chromosome in hybrids, we have utilized a retrovirus carrying a drug resistance marker (neo, conferring G418 resistance) and pseudotyped into a primate retrovirus helper envelope (GaLV, gibbon ape leukemia virus). When human cells are infected with such a virus, insertion occurs randomly in different chromosomes. Fusion of the infected cells and selection for G418-resistant hybrids result in a panel of hybrids retaining different human chromosomes in the presence of the selective drug (i.e., G418). We prepared a panel of 50 such hybrids between infected MNNG-HOS cells, carrying the activated met oncogene, and NIH3T3 mouse cells. Hybrids exhibiting transformed morphology were screened with met-specific probes and found to contain the activated met oncogene and, in some cases, the normal met gene. Screening of morphologically normal hybrids revealed two which contained only the normal met allele. Cytological analysis (Dr. J. Testa, Univ. of Maryland, Baltimore County) revealed that the apparently normal met allele was carried on a chromosome which appeared to contain a 1:7 translocation. Since the original HOS cell line prior to met activation contained a Tr 1:7, and the activated met oncogene consists of a fusion between met sequences and sequences from chromosome 1, this suggests that the translocation itself was not responsible for the met activation, and that additional MNNG-induced changes were apparently involved. The technique used here of making specific chromosomes using retroviruses carrying drug resistance markers, should be of great value in the analysis of both the structure and function of specific chromosomes involved in a variety of human neoplasms.

Publications:

O'Hara, B. M., Klinger, H. P. and Blair, D. G.: Levels of fos, ets-2 and myb RNAs correlate with tumorigenicity of HeLa/human fibroblast hybrids. In Puett, D., Ahmad, F., Black, S., Lopez, D. M., Melner, M. H., Scott, W. A. and Whelan, W. J. (Eds.): Advances in Gene Technology: Molecular Biology of the Endocrine System. Cambridge, Cambridge University Press, 1986, pp. 136-137.

O'Hara, B. M., Oskarsson, M., Tainsky, M. A. and Blair, D. G.: Mechanism of activation of human ras genes cloned from a gastric adenocarcinoma and a pancreatic carcinoma cell line. Cancer Res. (In Press)

Papas, T. S., Blair, D. G., Fisher, R. J., Watson, D. K., Sacchi, N., Fujiwara, S., Bhat, N. and Ascione, R.: The ets genes. In Reddy, E. P., Curran, T. and Skalka, A. (Eds.): The Oncogene Handbook. New York, Elsevier (In Press)

Park, M., Dean, M., Cooper, C. S., Schmidt, M., O'Brien, S. J., Blair, D. G. and Vande Woude, G. F.: Mechanism of met oncogene activation. Cell (In Press)

Patents:

None

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01CP05440-02 LMO

PERIOD COVERED

October 1, 1985 to September 30, 1986

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Site-Directed Mutagenesis of ras Oncogenes

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	D. J. Clanton	Senior Staff Fellow	LMO	NCI
Others:	T. Y. Shih	Research Chemist	LMO	NCI
	L. S. Ulsh	Microbiologist	LMO	NCI

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TOTAL MAN-YEARS:

1.2

PROFESSIONAL:

1.0

OTHER:

0.2

CHECK APPROPRIATE BOX(ES)

- (a) Human subjects (b) Human tissues (c) Neither
- (a1) Minors
- (a2) Interviews

SUMMARY OF WORK (Use standard un-reduced type. Do not exceed the space provided.)

Oligonucleotide-directed, site-specific mutagenesis is used to dissect the biochemical basis of oncogenic activation and of enzymatic activity of the ras oncogene. Studies are directed toward an understanding of the interrelationship between the known properties of the ras gene product. Mutagenesis of the ras oncogene in specific regions of the protein has been designed to explore the active center which is believed to be responsible for these properties.

We have constructed several point mutations at the GTP binding site of p21. Both lysine and tyrosine mutations of asparagine-116 abolish GTP binding and transforming activities of p21. These activities are retained by mutations at the 117 or 118 position. Both 116 mutant p21s, when overproduced in *E. coli*, are apparently devoid of GTP binding and autokinase activities. These mutant DNAs do not transform NIH3T3 cells in a focus-forming assay, and cells that are transfected with 116 mutants incorporate the exogenous v-ras sequences, express p21, and are contact-inhibited. In contrast to competent clones, defective p21 proteins are not autophosphorylated *in vivo*, indicating loss of biochemical activity. Mutations of the glycine residues within the ATP/GTP-binding consensus sequences, GXXXXGK, also greatly affected the GTP binding activity. These studies indicate that the GTP binding domain of p21 is crucial for its cellular function.

PROJECT DESCRIPTIONNames, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

D. J. Clanton	Senior Staff Fellow	LMO	NCI
T. Y. Shih	Research Chemist	LMO	NCI
L. S. Ulsh	Microbiologist	LMO	NCI

Objectives:

The objective of this project is to study the biochemical basis of oncogenic activation and of enzymatic activity of the ras oncogenes and their products by site-directed mutagenesis. Mutant proteins developed in this way can be expressed in a bacterial expression system, and the comparative biochemistry of the mutant proteins studied. The oncogenicity of the mutant viruses thus generated can be assessed by in vitro NIH3T3 cell transfection and by in vivo animal tumor formation.

Methods Employed:

Oligonucleotides of 17 bases containing single base changes from the v-ras^H gene are used for site-directed mutagenesis as described by Zoller and Smith (DNA 3: 479-488, 1984). An SstII site was inserted at the SmaI site of M13mp10 and the 1.1 kb SstII/XbaI fragment from the Harvey murine sarcoma virus genome clone, pH-1, was cloned into the new construct of M13. Single-stranded DNA from this phage was prepared and used as the template for mutagenesis. Phage containing the mutations are identified by plaque hybridization using ³²P-labeled oligonucleotides, and the DNA sequence confirming the mutation is performed by the chain termination method of Sanger et al. (Proc. Natl. Acad. Sci. USA 74: 5463-5467, 1977). The SstII/XbaI fragment containing the mutation was then used to reconstruct the pH-1 clone. The internal 0.88 kb HindIII fragment containing the genome for p21 can be cloned from the mutated phage DNA into the bacterial expression vector, pJL6, (Science 221: 858-860, 1983).

We have also constructed a plasmid which contains the entire genome of Harvey murine sarcoma virus linked to the neomycin resistance gene of pSV2-neo, which confers resistance to the drug G418 (J. Mol. Appl. Genet. 1: 327-341, 1982). This system will allow us to select for rastransfected cells by a phenotype unrelated to the transforming ability of the ras gene. Therefore, mutations which may abrogate the ability to transform cells can be easily detected and isolated.

A number of other laboratory techniques are utilized for these studies. ras genes (as well as other DNA fragments) are isolated by gel electrophoresis and identified by blot hybridization. Proteins expressed by this system and purified by column chromatography and analyzed by polyacrylamide gel electrophoresis and Western blotting show the GTP binding domain of p21 is crucial for its cellular function. Biochemical studies of the mutated ras proteins employ filter binding assays and immunoprecipitation by specific monoclonal

antibodies. Oncogenicity of the mutated ras genes can be measured by transfection of NIH3T3 cells (J. Virol. 26: 291-298, 1978).

Major Findings:

The ras gene family encodes a group of closely-related 21,000 dalton (p21) proteins. These proteins of 188 or 189 amino acid residues are highly conserved in sequence, which suggests not only that most of their structure is functionally significant, but also that cellular ras genes must play an essential role in growth and development. There are three known biochemical activities associated with p21. The p21 of all ras genes binds GTP or GDP with high affinity and displays a low GTPase activity, which is further reduced by oncogenic activation. Additionally, the p21s of the murine sarcoma viruses (Harvey and Kirsten MSV) exhibit an autokinase activity due to substitution of a threonine residue for the alanine-59 of c-ras genes at the autophosphorylation site. Like many other GTP binding proteins (or G proteins as they are called), p21 is found on the inner surface of the plasma membrane. This has led to the suggestion that p21 may function as a signal transducer, relaying extracellular messages to the interior cellular effectors. The oncogenic p21, whose GTPase activity is reduced, may remain active much longer and thereby overstimulate the cell's regulatory mechanisms.

The p21 proteins of ras genes are highly conserved in primary amino acid sequences. There is also extensive sequence homology found between p21 and the family of G proteins which bind GTP/GDP, and it is likely that these regions represent a part of the GTP binding site. The present experiments are designed to delineate the site critical for binding GTP/GDP and to evaluate the biological consequences of nucleotide binding to ras gene function. We have constructed mutations by oligonucleotide-directed, site-specific mutagenesis which result in single amino acid substitutions of p21, with drastic changes in side chains at the site which appears to interact with the guanine base. From the three-dimensional structure determined by X-ray crystallography for the G protein EF-Tu (EMBO J. 4: 2385-2388, 1985; Science 230: 32-36, 1985), the homologous Asn-116 and Asp-119 of p21 should have several critical interactions with the guanine ring. The present mutations of p21, changing Asn-116 into either a lysine or a tyrosine residue, result in proteins which have apparently lost their ability to bind nucleotides. These mutant p21 proteins, when expressed in E. coli, do not show any binding activities, even at nucleotide concentrations 10,000-fold of that of the K_d of wild-type p21, indicating that the binding affinity has decreased more than 10,000 times. Loss of p21 activities was also evident when mutant ras genes were introduced into NIH3T3 cells by transfection. No phosphorylated p21 was observed in both of the 116 residue mutations due to lack of autokinase activities. Mutations at the adjacent 117th or 118th positions do not abolish p21 GTP binding and its associated activities. The present finding suggests that the basic structure of the GTP binding site is conserved between p21 and EF-Tu.

Another question we have addressed is the biological significance of GTP binding of p21 to the ras gene function. In the present study, we have found that mutations of Asn-116 to either lysine or tyrosine resulted in loss of transforming activity of the proviral DNA in a focus-forming assay using NIH3T3 cells, while all other mutations (i.e., Lys-117, Cys-118), which retain their GTP binding

ability, were still capable of cellular transformation. G418-resistant cell clones were obtained by cotransfection with the positive selectable marker, pSV2-neo DNA, and mutant or wild-type DNA. The 116K and 116Y mutant transformants remained contact-inhibited, while all others were morphologically transformed. We interpret these results to mean that p21 GTP binding is crucial for the cellular function of ras genes.

We have also made mutations changing the autophosphorylation site of v-ras, threonine-59, into alanine and serine. As expected, the alanine mutant cannot be phosphorylated, and the serine mutant exhibited a reduced autokinase activity, which was reflected in a 10- to 100-fold decrease in GTP binding affinity of the serine mutant. These results are consistent with the juxtaposition of this threonine residue with the gamma-phosphate of GTP.

Our current construct of p21 for expression in a bacterial system is a fusion protein replacing the first 4 amino acid codons of p21 with the first 14 codons of the bacteriophage lambda cII gene. The fusion point precedes the region of p21 which contains the consensus sequence of GXXXXGK (Gly¹⁰XXXXGly¹⁵Lys¹⁶ of p21) found in most ATP or GTP binding proteins. Our mutations changing these two glycine residues into valine greatly affect the ³H-GTP binding activity of p21, suggesting not only that there is a critical role for this sequence, but also that it comes in close proximity to the GTP binding site near Asn-116.

Proto-oncogenes and activated oncogenes apparently share essentially the same critical activity of GTP/GDP binding, and perhaps the purpose of proto-oncogene activation is to increase the potency of this basic function by either mutations that decrease the GTP hydrolyzing activity, or alternatively, by mass action to increase the protein level by enhanced transcription, for example, by viral LTR or other cellular enhancers. These studies underscore the importance of the GTP binding site for the transformation activity of ras oncogenes, and may provide further impetus to explore means of cancer intervention by interfering with this critical activity of p21 proteins.

Publications:

Clanton, D. J., Hattori, S. and Shih, T. Y.: Mutations of the p21 ras gene which abolish guanine nucleotide binding and transformation. Proc. Natl. Acad. Sci. USA (In Press)

Shih, T. Y., Clanton, D. J., Hattori, S., Ulsh, L. S. and Chen, Z.-Q.: Structure and function of p21 proteins: Biochemical, immunochemical and site-directed mutagenesis studies. In Colburn, N. H., Moses, H. L., Stanbridge, E. J. and Fox, C. F. (Eds.): Growth Factors, Tumor Promoters, and Cancer Genes. New York, Alan R. Liss, Inc. (In Press)

Patents:

None

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01CP05441-02 LMO

PERIOD COVERED

October 1, 1985 to September 30, 1986

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Characterization of the Gene Products of the c-myc Locus and the c-ets Locus

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)

PI:	R. J. Fisher	Expert	LMO	NCI
Others:	S. Fujiwara	Visiting Fellow	LMO	NCI
	N. Bhat	Visiting Fellow	LMO	NCI
	T. S. Papas	Chief	LMO	NCI

COOPERATING UNITS (if any)

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Laboratory of Molecular Oncology

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Carcinogenesis Regulation Section

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TOTAL MAN-YEARS:

1.2

PROFESSIONAL:

1.2

OTHER

0.0

CHECK APPROPRIATE BOX(ES)

- (a) Human subjects (b) Human tissues (c) Neither
- (a1) Minors
- (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The protein(s) encoded by the c-myc locus and the c-ets locus has been examined in human cell lines and will be isolated from those cell lines which show these proteins to be amplified. To probe these proteins, polyclonal or monoclonal antibodies were prepared against peptides representing derived amino acid sequences from the myc or ets DNA sequence.

The ets-2 and myc proteins have been localized by subcellular fractionation. The ets-2 protein shows primarily nuclear localizations, while the myc proteins, in addition to showing nuclear localizations, also show cytoplasmic localization.

The ets-2 p56 protein and the myc p58, p60 (cytoplasmic) and p80 (cytoplasmic) proteins have been partially or completely purified (myc p60). The N-terminal amino acid sequence of the myc p60 (cytoplasmic) has been obtained and is found not to correspond to the derived amino acid sequence of myc. This new protein may correspond to a family of myc-related proteins.

PROJECT DESCRIPTIONNames, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

R. J. Fisher	Expert	LMO	NCI
S. Fujiwara	Visiting Fellow	LMO	NCI
N. Bhat	Visiting Fellow	LMO	NCI
T. S. Papas	Chief	LMO	NCI

Objectives:

The objective of this work is to characterize the protein products of the human cellular proto-oncogenes, c-myc, c-ets-1, and c-ets-2.

Methods Employed:

Biological Materials: Human tumor cells, COLO 320 DM, are used as an enriched source of the c-myc and c-ets-2 proteins. Thymus (mouse or calf) is used as an enriched source of c-ets-1 and of c-ets-2. Mouse cells infected with vectors containing two or three exon constructs of c-myc are used as positive controls for the identification of the c-myc protein.

Protein Isolation: Cells and tissues are subfractionated into either nuclear or cytoplasmic components. The c-myc and c-ets-2 are extracted from nuclei and purified to homogeneity by fast protein liquid chromatography (FPLC) (anion exchange) and high pressure liquid chromatography (Aquapore 300 reverse phase HPLC).

Protein Identification: Monoclonal or polyclonal antibodies prepared against peptides derived from the DNA sequences of myc or ets are used to assay the purification of the corresponding cellular proteins. Peptide mapping (one-dimension [CnBr or S. aureus protease] or trypsin maps by HPLC) is used to determine the relatedness of various radiolabelled proteins identified with the antibodies. Structural analysis is carried out by amino acid sequencing with the gas-phase amino acid sequences.

Protein Sequence Analysis: The NIH DEC10 computer or the NCI supercomputer is used to analyze the amino acid sequences obtained.

Major Findings:

Identification of the c-myc Proteins: A monoclonal antibody prepared against an N-terminal peptide of c-myc identified several proteins by immunoprecipitation of ³⁵S-methionine, pulse-labelled COLO 320 DM cells. Two of these proteins, p60 (major) and p58 (minor), are found in mouse cells infected with either two or three exon human c-myc constructs. The one-dimensional peptide maps of the p60 and p58 from COLO cells and the p60 from transfected mouse cells are exactly the same. We conclude that these proteins represent c-myc and that the reading frame starts with the second exon and not with the first exon reading frame.

Localization of the c-myc Proteins: Using standard subcellular fractionation techniques and immunoprecipitation techniques we find that the p58 myc is exclusively nuclear and the p60 myc is distributed between the cytoplasm and nuclei. Additionally, a p80 protein, specifically recognized by the N-terminal monoclonal antibody, is primarily cytoplasmic.

Purification of the c-myc Proteins: All of the c-myc proteins identified with the N-terminal monoclonal antibody have been purified to homogeneity. The nuclear p58 copurifies with an intermediate filament protein, vimentin. We think that the p58 myc protein is the same size and has a similar isoelectric point as vimentin, but represents only a very small percentage (<0.1%) of the 58-kilodalton proteins. For this reason, other than demonstrating its presence, we have not been able to do much with it.

The cytoplasmic p60 (perhaps one of several) has been purified to homogeneity and the N-terminal amino acid sequence determined. The amino acid sequence did not correspond to the human c-myc sequence, but instead demonstrated a weak (30-50%) homology to a 5' domain of the human N-myc protein. This finding suggests that myc may be a member of a gene family with several members which can also be recognized by our monoclonal antibody.

Identification of the ets-2 Protein: The use of a polyclonal antibody prepared against a highly conserved region of the ets-2 (a region found in all three ets-2 transcripts) identified the p56 protein in COLO cells or mouse thymus. Additionally, polyclonal antibodies prepared against the v-ets-2-expressed protein also immunoprecipitate a p56 protein. The one-dimensional peptide maps of the p56 protein found with the anti-peptide antibody and the p56 protein found with the antibody against the v-ets-expressed protein are exactly the same.

Localization of the ets-2 Protein: Using standard subcellular fractionation techniques, immunoblotting and immunoprecipitation techniques, we found the ets-2 p56 to be localized to the nucleus. This contrasts with the findings of Ghysdoel et al. (Proc. Natl. Acad. Sci. USA 83: 1714-1718, 1986), who demonstrated that the chicken p56 is found in the cytoplasm. However, in chickens there is only one transcript and ets-1 and ets-2 are in the same transcript, so that these factors may affect the localization.

Purification of the ets-2 Protein: The ets-2 protein has been partially purified from the nuclei of COLO 320 cells. The nuclear extracts are initially fractionated on FPLC anion exchange and then on reverse phase HPLC. Since we do not know the N-terminal sequence of the ets-2 protein, we will isolate the fragment containing the epitope for our peptide antibody by techniques involving isolation of peptides by HPLC and amino acid sequencing by gas phase. We have a bacterially-expressed protein which contains our peptide epitope. I have successfully cut it with CnBr and isolated a single peptide which reacted with our peptide antibody. The N-terminal amino acid sequence of this fragment exactly corresponded to the amino acid sequence predicted from the cDNA clone of this region.

Publications:

Ascione, R., Sacchi, N., Watson, D. K., Fisher, R. J., Fujiwara, S., Seth, A. and Papas, T. S.: Oncogenes: Molecular probes for clinical application in malignant diseases. Gene Anal. Tech. 3: 25-39, 1986.

Papas, T. S., Blair, D. G., Fisher, R. J., Watson, D. K., Sacchi, N., Fujiwara, S., Bhat, N. and Ascione, R.: The ets genes. In Reddy, E. P., Curran, T. and Skalka, A. (Eds.): The Oncogene Handbook. New York, Elsevier (In Press)

Patents:

None

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01CP05442-02 LMO

PERIOD COVERED

October 1, 1985 to September 30, 1986

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Involvement of c-ets in the Pathogenesis of Human Leukemias

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	N. Sacchi	Visiting Associate	LMO	NCI
Others:	D. K. Watson	Senior Staff Fellow	LMO	NCI
	T. S. Papas	Chief	LMO	NCI

COOPERATING UNITS (if any)

Department of Cell Biology and Genetics, Erasmus University, Rotterdam, The Netherlands (A. Hagemeijer); Department of Neurogenetics, Eleanor Roosevelt Cancer Institute, Denver, CO (H. D. Drabkin)

LAB/BRANCH

Laboratory of Molecular Oncology

SECTION

Carcinogenesis Regulation Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701-1013

TOTAL MAN-YEARS:

1.1

PROFESSIONAL:

1.1

OTHER:

0.0

CHECK APPROPRIATE BOX(ES)

- (a) Human subjects (b) Human tissues (c) Neither
- (a1) Minors
- (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Human ets genes are located on chromosome regions of interest to human cancer, as well as to human genetics. These regions are the 11q23-24 for ets-1 and 21q22.3 for ets-2. Cancer-specific chromosome abnormalities involving these regions are found mainly in leukemias, lymphomas and solid tumors (Ewing's sarcoma). Using panels of somatic cell hybrids, we have localized the ets genes relative to a number of chromosome breakpoints characteristic of specific translocations occurring in neoplastic cells. We have found the ets-1 gene locates in a very narrow region of chromosome 11, between the breakpoint of the t(4;11)(q21;q23) of an acute leukemia and the t(11;22)(q24;q12) of a Ewing's sarcoma. The ets-2 gene on chromosome 21 is bracketed by the breakpoints of a t(8;21)(q22;q22) of an acute myelogenous leukemia (AML-M2) and a t(21;22)(q22;q11) of a chronic myeloid leukemia. Both ets-1 and ets-2 were not found to be rearranged, using probes representing the 3' regions of the genes, regardless if the genes were transposed or not. Therefore, it remains to be defined whether these genes are directly involved in some of the breakpoints, or if they are in close proximity to the newly formed chimeric junctions. The ets-2 gene maps in the particular region of chromosome 21 that, when trisomic, confers the Down's syndrome (DS) phenotype. Restriction fragment length polymorphisms (RFLPs) for the ets-2 gene (*Msp* I and *Taq* I) have been used for a genetic population study aimed at the prevention of this syndrome. DS individuals, with an extra copy of chromosome 21, develop an Alzheimer-like disease. One form of Alzheimer's disease seems to have an autosomal dominant inheritance, strongly suggesting the involvement of a responsible gene on chromosome 21. This fact provides a rationale for using ets-2 RFLPs in searching for the Alzheimer's disease gene in high-risk families.

PROJECT DESCRIPTIONNames, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

N. Sacchi	Visiting Associate	LMO	NCI
D. K. Watson	Senior Staff Fellow	LMO	NCI
T. S. Papas	Chief	LMO	NCI

Objectives:

(a) To study the role of the cellular homologs of the v-ets gene in the etiology of human neoplasia as it relates to chromosome abnormalities associated with specific malignant disorders.

(b) To use the restriction fragment length polymorphisms (RFLPs) to search for familial cancers, as well as for inherited human genetic diseases, e.g., Alzheimer's disease.

Methods Employed:

Molecular analysis of chromosome breakpoints by c-ets probes and other probes. Northern blot analysis of ets genes in leukemic cell lines. Use of RFLPs in Southern blot experiments aimed to detect specific haplotypes correlated to Down's syndrome.

Major Findings:

Most of the chromosomal abnormalities seen in neoplastic cells are believed to result from the clonal proliferation of cells in which a chromosomal abnormality has arisen by somatic mutation. Several specific nonrandom chromosomal abnormalities have been associated with well-defined malignancies, as in the cases of the Philadelphia chromosome derived from the translocation t(9;22) in chronic myelogenous leukemia, the t(15;17) in acute promyelocytic leukemia (ANLL-M3), and the t(8;14) associated with human Burkitt's lymphoma.

(a) The current investigations have allowed us to localize the ets genes relative to a number of chromosome breakpoints characteristic of specific translocations occurring in neoplastic cells. We have found the ets-1 gene locates on a very narrow region of chromosome 11, between the breakpoints of the t(4;11)(q21;q23) of an acute leukemia and the t(11;22)(q24;q12) of a Ewing's sarcoma. The ets-2 gene on chromosome 21 is inactivated by the breakpoints of a t(8;21)(q22;q22) of an acute myelogenous leukemia (AML-M2) and a t(21;22)(q22;q11) of a chronic myeloid leukemia. Both ets-1 and ets-2 were not found to be rearranged using probes representing the 3' regions of the genes, regardless if the genes were transformed or not. Therefore, it remains to be defined whether these genes are directly involved in some of the breakpoints, or if they are in close proximity to the newly formed chimeric junctions.

(b) RFLPs for ets-2 genes have been determined with the Msp I and Taq I enzymes. These RFLPs have been used in combination with the RFLPs for other chromosome 21-specific probes.

Publications:

Ascione, R., Sacchi, N., Watson, D. K., Fisher, R. J., Fujiwara, S., Seth, A. and Papas, T. S.: Oncogenes: Molecular probes for clinical application in malignant diseases. Gene Anal. Tech. 3: 25-39, 1986.

LeBeau, M. M., Rowley, J. D., Sacchi, N., Watson, D. K., Papas, T. S. and Diaz, M. O.: Hu-ets-2 is translocated to chromosome 8 in the t(8;21) of acute myelogenous leukemia. Cytogenet. Cell Genet. (In Press)

Papas, T. S., Blair, D. G., Fisher, R. J., Watson, D. K., Sacchi, N., Fujiwara, S., Bhat, N. and Ascione, R.: The ets genes. In Reddy, E. P., Curran, T. and Skalka, A. (Eds.): The Oncogene Handbook. New York, Elsevier (In Press)

Papas, T. S., Watson, D. K., Sacchi, N., O'Brien, S. and Ascione, R.: The cellular ets genes: Molecular biology and clinical implications in human leukemias. Cancer Invest. (In Press)

Papas, T. S., Watson, D. K., Sacchi, N., O'Brien, S. and Ascione, R.: The cellular ets genes: Molecular probes in human neoplasia. In Proceedings of International Advanced Course of Human Pre-Leukemia (In Press)

Papas, T. S., Watson, D. K., Sacchi, N., O'Brien, S. J. and Ascione, R.: The mammalian ets genes: Two unique chromosomal locations in cat, mice and man and novel translocated position in human leukemias. In Hagenbeek, A. and Lowenberg, B. (Eds.): Minimal Residual Disease in Acute Leukemia: 1986. Dordrecht/Boston, Martinus Nijhoff Publishing, 1986, pp. 23-42.

Papas, T. S., Watson, D. K., Sacchi, N., O'Brien, S. J. and Ascione, R.: Molecular evolution of ets genes from avians to mammals and their cytogenetic localization to regions involved in leukemia. In Papas, T. S. and Vande Woude, G. F. (Eds.): Gene Amplification and Analysis: Oncogenes. New York, Elsevier Vol. 4 (In Press)

Sacchi, N., Watson, D. K. and Papas, T. S.: Ets genes in human acute leukemias. In Puett, D., Ahmad, F., Black, S., Lopez, D. M., Melner, M. H., Scott, W. A. and Whelan, W. J. (Eds.): Advances in Gene Technology: Molecular Biology of the Endocrine System. Cambridge, Cambridge University Press, 1986, pp. 142-143.

Sacchi, N., Watson, D. K., van Kessel, A. H. M. G., Hagemeyer, A., Drabkin, H. D., Patterson, D. and Papas, T. S.: Hu-ets-1 and Hu-ets-2 genes are transposed in acute myeloid leukemias with (4;11) and (8;21) translocations. Science 231: 379-382, 1986.

Watson, D. K., Sacchi, N., McWilliams-Smith, M. J., O'Brien, S. J. and Papas, T. S.: The avian and mammalian ets genes: Molecular characterization, chromosome mapping, and implication in human leukemia. Anticancer Res. (In Press)

Patents:

None

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01CP05443-02 LMO

PERIOD COVERED

October 1, 1985 to September 30, 1986

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Oncogene Expression During Cell Differentiation and Development

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)

PI:	R. J. Fisher	Expert	LMO	NCI
Others:	N. K. Bhat	Visiting Fellow	LMO	NCI
	T. S. Papas	Chief	LMO	NCI
	R. Ascione	Research Chemist	LMO	NCI
	S. Fujiwara	Visiting Fellow	LMO	NCI

COOPERATING UNITS (if any)

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NCI, NIH, Frederick, Maryland 21701-1013

TOTAL MAN-YEARS:

1.5

PROFESSIONAL:

1.5

OTHER:

0.0

CHECK APPROPRIATE BOX(ES)

- (a) Human subjects (b) Human tissues (c) Neither
- (a1) Minors
- (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Analysis of c-ets gene expression during spermatogenesis, thymus development and during compensatory growth of liver indicates that (i) ets-2 gene expression is linked to cell proliferation and occurs before DNA synthesis, (ii) ets-2 gene expression may be regulated during the course of development, (iii) both ets-1 and ets-2 genes are differentially regulated, and (iv) ets genes may belong to the nuclear family of oncogenes. In mice, the ets-2 gene is transcribed as a major mRNA species of 4.2 kb and expressed in most of the tissues examined. The ets-1 gene is transcribed as multiple mRNA species sized 7.5 kb, 2.4 kb and 1.7 kb. The product of ets-2 appears to be preferentially expressed as a protein of 56 Kd in size, and has been identified as a putative ets-2 gene protein which is expressed at much higher levels in the thymus. The role of ets gene products in cell proliferation and differentiation is under investigation.

Human myc genes containing entire coding sequences or only the second and third exons have been expressed under the control of the metallothionein promoter, using the bovine papillomavirus (BPV) vector system. Permanent cell lines expressing human myc proteins have been established. Analysis of human myc gene products in these cell lines indicates that (i) myc gene products enhance BPV-induced transformation, (ii) 62-64-Kd human myc protein is made either when all three exons are present or only the second and third exons are present, (iii) human myc protein expressed in mouse cells is mainly compartmentalized in the nucleus, (iv) human myc protein is inducible with heavy metal ions, and (v) though the myc gene is present on an episome in the cell, it appears to be subject to a similar regulatory control mechanism(s) like those controlling the endogenous c-myc gene.

PROJECT DESCRIPTIONNames, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

R. J. Fisher	Expert	LMO	NCI
N. K. Bhat	Visiting Fellow	LMO	NCI
T. S. Papas	Chief	LMO	NCI
R. Ascione	Research Chemist	LMO	NCI
S. Fujiwara	Visiting Fellow	LMO	NCI

Objectives:

To determine the role of nuclear oncogene products, specifically the role of the ets gene products, in cell proliferation and differentiation, and to understand the molecular mechanisms involved in the regulation of ets gene expression; and to develop a mammalian expression vector system to express genes of special interest.

Methods Employed:

1. Isolation of nucleic acids, and Northern and Southern analyses. Isolation of high molecular weight DNA, total RNA and poly A⁺ RNA, nucleic acid fractionation on agarose gels, transfer of nucleic acids to membranes, probe preparation, hybridization and washing of filters were done as described by Maniatis et al. (Molecular Cloning, A Laboratory Manual, 1982).
2. Cloning. Restriction enzyme digestion, isolation of DNA fragments from gels by electroelution and elutip-d column chromatography, ligation, transformation of competent cells by plasmids, plasmid isolation and characterization were carried out as described by Maniatis et al. Human ets-specific clones, pH33, pRD700, pKE600 and pEH, v-ets, mouse ets-specific clones, M-ets-2 and M-ets-1 were provided by Dr. D. K. Watson of this Laboratory. Appropriate ets DNA fragments were subcloned in pGEM vectors to get a higher yield of plasmids and to prepare labeled riboprobes.
3. Construction of pMT bovine papillomavirus (BPV) H-myc-3 and pMT BPV H-myc-2. The Sall-BamHI fragment was isolated from pMT5872 and pMT3600 and ligated to the Sall and BamHI fragment containing the entire BPV genome. A 2.0 Kbp BamHI fragment containing the entire human metallothionein gene was then inserted into the unique BamHI site.
4. Establishment of permanent cell lines expressing human myc genes. pMT BPV H-myc-3, pMT BPV H-myc-2 and pBPV MTX (kindly provided by Dr. G. N. Pavlakis, Litton Bionetics, Inc.) DNA were transfected into C127 cells by the calcium precipitation method. Individual Cd²⁺ resistant cells were selected and clonally expanded. Cells were then grown in the presence of 20 μ m CdCl₂. Human myc polypeptide species were characterized by Western blot and immunoprecipitation analyses using monoclonal antisera raised against different peptide regions of predicted human myc protein sequences. Human myc DNA and

RNA in these permanent cell lines were characterized by Southern and Northern blot analyses, respectively.

5. Partial hepatectomy. Partial hepatectomy was done by excising out two-thirds of the liver according to the method of Higgins and Anderson (Arch. Pathol. Lab. Med. 12: 186-202, 1931). Animals were sacrificed at appropriate times and RNA was isolated from the liver as described by Chirgwin et al. (Biochemistry 18: 5294-5299, 1979). c-onc transcripts were characterized by probing with nick-translated c-onc probes.

Major Findings:

1. Northern analysis of poly A⁺ RNA from mouse testes, brain, liver, thymus, and spleen indicated that the ets-2 gene is transcribed as 4.2 kb mRNA (major species), as compared to multiple species found in human cell lines.
2. ets-2 transcripts are abundant in young testes and young thymus, which are highly proliferative tissue; whereas they are present in very low amounts in terminally-differentiated, nonproliferating adult tissues.
3. Kinetics of the appearance of ets-2 transcripts during compensatory growth of the liver suggests that the ets-2 gene is transcribed at the highest level before cells enter the S phase of the cell cycle, i.e., DNA synthesis. However, the induction of ets-2 mRNA follows the induction of myc mRNA.
4. Using antisera directed against an ets-2-specific oligopeptide, a protein of 56 Kd in size has been detected in young testes and young thymus, but not in adult testes. This correlates very well with the level of ets-2 mRNA in these tissues.
5. The ets-1 gene is transcribed into multiple mRNA species (7.5 kb, 2.4 kb, and 1.7 kb) and appears to be expressed preferentially in thymus. The complexity of these transcripts appears to be similar to that found in human cell lines. The kinetics of induction of ets-1 mRNA appears to be different from that of ets-2 mRNA, suggesting that these two genes are regulated independently.
6. Using monoclonal antibodies raised against oligopeptides from three different regions of predicted human myc sequence proteins in the size range of 62-64 KDa, polypeptides have been detected both by Western blot and immunoprecipitation analyses. These polypeptides are transfection-specific since no protein bands in that size range have been detected with these monoclonal antibodies in cells transfected with vectors alone. Human myc mRNA and proteins in these cells are inducible with heavy metal ions. Though the mRNA size is different in cells transfected with pMT BPV H-myc-3 (all three exons), compared to pMT BPV H-myc-2 (second and third exons), the same size proteins are expressed in these cell lines. Fingerprint analysis of the 62-64 KDa polypeptide species obtained from cells transfected with pMT BPV H-myc-3 or pMT BPV H-myc-2 shows similarity, indicating that both species are identical.
7. Human myc proteins expressed in mouse cells are mainly localized in the nucleus.

Publications:

Papas, T. S., Blair, D. G., Fisher, R. J., Watson, D. K., Sacchi, N., Fujiwara, S., Bhat, N. and Ascione, R.: The ets gene. In Reddy, E. P., Curran, T. and Skalka, A. (Eds.): The Oncogene Handbook. New York, Elsevier (In Press)

Patents:

None

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01CP05483-01 LMO

PERIOD COVERED

October 1, 1985 to September 30, 1986

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

RNA Processing and Gene Control - the rnc Operon of RNaseIII

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	D. L. Court	Research Biologist	LMO	NCI
Others:	H. E. Takiff	Guest Researcher	LMO	NCI
	R. J. Fisher	Expert	LMO	NCI

COOPERATING UNITS (if any) Program Resources, Inc., Frederick, MD (T. Patterson); Dept. of Gen. & Mol. Biol., Cent. de Invest. y de Estudios Avanzados Del IPN, Mexico City, Mexico (G. Guarneros, P. Guzman); E. I. duPont de Nemours & Co., Inc., Wilmington, DE (S. E. Bear); Dept. of Biochem., State U. of NY, Stony Brook, NY (P. E. March)

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1.0

PROFESSIONAL:

1.0

OTHER:

0.0

CHECK APPROPRIATE BOX(ES)

- (a) Human subjects (b) Human tissues (c) Neither
 (a1) Minors
 (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

RNaseIII is a double-strand specific endoribonuclease that has different functions in E. coli. It processes rRNA precursors for efficient maturation into ribosomes. It processes some mRNAs either to activate gene expression or to reduce gene expression. It regulates mRNA degradation.

The int gene of phage λ is transcribed from two promoters yielding different mRNA transcripts. Int expression from one is reduced by RNaseIII; from the other, expression is enhanced. In both cases, control of expression by RNaseIII occurs from a single site beyond the gene. This form of control is named retroregulation. The site present on the RNA is able to form a special stem and loop structure that is recognized by RNaseIII.

In order to understand how RNaseIII levels in the cells are modulated, its gene in E. coli, rnc, has been cloned on λ vectors and on pBR322 plasmid. Sequence analysis indicates a second gene in an operon with rnc. This gene produces a protein with significant homologies to the yeast ras genes and is called era (E. coli ras). Both rnc and era have been placed on expression vectors and their proteins have been purified and antibodies made. Preliminary evidence suggests that era is an essential gene in E. coli and that the protein binds GTP.

PROJECT DESCRIPTIONNames, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

D. L. Court	Research Biologist	LMO	NCI
H. E. Takiff	Guest Researcher	LMO	NCI
R. J. Fisher	Expert	LMO	NCI

Objectives:

RNaseIII exerts retroregulation on λ int gene expression. To understand RNaseIII levels and the degree to which they can modulate gene expression by RNA processing, RNaseIII's own gene regulation must be studied. How does cell growth and global regulatory factors like cAMP, ppGpp, and others affect rnc gene expression? Antibodies to RNaseIII have been made and they will be used to measure RNaseIII levels in cells. Transcriptional activity will be measured both by gene fusions to lacZ and directly by mRNA labeling and hybridization studies.

Similar analyses of era (*E. coli ras*) will be carried out to examine how its expression is controlled as part of an operon with rnc. Mutations in both rnc and era will be isolated. Since era appears to be essential for cell growth, conditionally lethal mutations will be sought first. The effect of these mutations on cell physiology under nonpermissive conditions will be examined.

E. coli genes of related function are frequently joined in operons. The relationship between the functions of rnc and era will be examined. Other laboratories will be examining the enzymological properties of the purified proteins, i.e., RNA binding and processing, GTP binding, etc.

Methods Employed:

Standard microbial, genetic, biochemical, and recombinant DNA techniques are used. RNaseIII is assayed by degradation of labeled poly A-U duplexes to TCA solubility. Using an HPLC column, both RNaseIII and the era product were purified from cells where the protein was produced by an expression vector, pTK23, at levels equal to 10-20% of total protein.

Antibodies will be used to detect endogenous cellular levels by both the ELISA assay and more rapidly by Western blot analysis.

RNA hybridizations to rnc and era genes will be detected using RNA probes made with in vitro transcription by T7 and/or SP6 polymerase.

Major Findings:

1. Retroregulation of λ int gene expression by RNaseIII has been extensively characterized (see Z01CP08718-07 LMO, 1985).

2. The rnc gene, encoding RNaseIII, has been cloned on λ and pBR322 vectors from genomic libraries of E. coli.
3. In our clones of rnc, a second gene, era, has been detected in the same operon. Sequence analysis indicates a relationship of this E. coli protein to yeast ras protein.
4. A complete genetic map of the rnc era region of the coli chromosome has been nearly completed using transposition by mini-tn10 and PI transduction to localize rnc era and nearby genetic markers: glyA, pdx, nadB, purI.
5. Insertion of the gene for chloramphenicol acetyl transferase into the middle of era on λ has been carried out. This mutant era cannot be transferred to the chromosome to replace the normal gene, indicating that era is essential to E. coli.

Publications:

Gussin, G., Hwang, J. J., Matz, K., Zuber, M. and Court, D.: Activation and repression of the λ P_{RE} promoter by cII protein. In Calendar, R. and Gold, L. (Eds.): Sequence Specificity in Transcription and Translation. UCLA Symposia on Molecular and Cellular Biology, New Series. New York, Alan R. Liss, Inc., 1985, Vol. 30, pp. 229-238.

Montanez, C., Bueno, J., Schmeissner, U., Court, D. L. and Guarneros, G.: Mutations of bacteriophage lambda that define independent but overlapping RNA processing and transcription termination sites. J. Mol. Biol. (In Press)

Sisk, W. P., Chirikjian, J. G., Lautenberger, J., Jorcyk, C., Papas, T. S., Berman, M., Zagursky, R. and Court, D. L.: A vector for selection and expression of gene segments: Expression of an HTLV-I envelope gene segment. Gene (In Press)

Tsugawa, A., Kurihara, T., Zuber, M., Court, D. L. and Nakamura, Y.: E. coli NusA protein binds in vitro to an RNA sequence immediately upstream of the boxA signal of bacteriophage lambda. EMBO J. 4: 2337-2342, 1985.

Patents:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05484-01 LMO

PERIOD COVERED

October 1, 1985 to September 30, 1986

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

The Cloned c-ets Gene of Sea Urchin and Its Expression During Embryogenesis

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	N. C. Kan	Senior Staff Fellow	LMO	NCI
Others:	Z.-Q. Chen	Visiting Fellow	LMO	NCI
	L. J. Pribyl	Biologist	LMO	NCI
	S. Fujiwara	Visiting Fellow	LMO	NCI
	R. J. Fisher	Expert	LMO	NCI
	R. Ascione	Research Chemist	LMO	NCI
	T. S. Papas	Chief	LMO	NCI

COOPERATING UNITS (if any)

Department of Biology, The Johns Hopkins University, Baltimore, MD
 (E. Moudrianakis)

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TOTAL MAN-YEARS:

1.8

PROFESSIONAL:

1.8

OTHER:

0.0

CHECK APPROPRIATE BOX(ES)

- (a) Human subjects (b) Human tissues (c) Neither
 (a1) Minors
 (a2) Interviews

SUMMARY OF WORK (Use standard un-reduced type. Do not exceed the space provided.)

The proto-oncogene, c-ets-2, of sea urchin (Lytechinus variegatus) has been molecularly cloned and sequenced. A comparison of the sequence of the c-ets-2 gene of sea urchin was made with the chicken retroviral homolog (v-ets) and the human cellular oncogene (Hu-ets-2). A remarkable conservation of these genes was noted; over 92% of the predicted amino acids of the sea urchin c-ets-2 was homologous to the viral oncogene. More than 94% of the predicted amino acids of sea urchin c-ets-2 matched with the human homolog Hu-ets-2; the highest homology thus far noted for such evolutionarily, widely-distributed oncogenes. The expression of messenger RNA was examined during sea urchin embryogenesis, using our cloned c-ets-2 DNA as a probe. It appears that the expression of the sea urchin c-ets-2 gene occurs early during embryonic development, peaking from 5 min to 17 hr post-fertilization, and declining markedly thereafter. The sea urchin mRNA identified by the cloned c-ets-2 probe was a unique species sized at 5.7-kb.

PROJECT DESCRIPTIONNames, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

N. C. Kan	Senior Staff Fellow	LMO	NCI
Z.-Q. Chen	Visiting Fellow	LMO	NCI
L. J. Pribyl	Biologist	LMO	NCI
S. Fujiwara	Visiting Fellow	LMO	NCI
R. J. Fisher	Expert	LMO	NCI
R. Ascione	Research Chemist	LMO	NCI
T. S. Papas	Chief	LMO	NCI

Objectives:

The determination of the evolutionary relationship of the proto-oncogene, ets, from invertebrates to man and its role and biological function in cellular growth and differentiation.

Methods Employed:

1. Isolation of DNA and RNA from sea urchin. Sea urchin eggs were fertilized and staged at various times during embryogenesis and grown in special sea-water medium. After harvesting, nucleic acids were fractionated and analyzed according to standard procedures (Molecular Cloning, A Laboratory Manual, 1982).
2. Identification and cloning of recombinant sea urchin proto-ets sequences. Restriction enzyme analysis, isolation of DNA fragments from gels by electroelution after agarose electrophoresis, elutip-d chromatography, ligation, transformation into competent cells by plasmid constructs, and preparation of partial sea urchin genomic DNA library. Plasmid preparation, isolation and labeled probes were performed as described by Maniatis *et al.* (1982). Sequence of DNA in M13 used as chain terminator for DNA sequencing method (Proc. Natl. Acad. Sci. USA 74: 5463-5467, 1977). Viral ets-2 DNA (1.28-kb) and the proto-oncogene homolog of Drosophila, D-ets-2 (0.4-kb) clones were nick-translated using ³²P-labeled nucleotides and were used to identify and detect the sea urchin proto-oncogene homologous to the ets-2 gene.
3. Expression of sea urchin proto-ets-2 gene. Isolation and selection of sea urchin mRNA by oligo dT column chromatography and analysis by Northern transfer (Northern blot) electrophoresis. Analysis of ets-2 related protein by SDS-PAGE and specific antibody against ets-2-predicted oligopeptide. Analysis by immunoelectrophoresis (Western blot) to identify the ets product during different stages of embryogenesis.

Major Findings:

Gene cloning and selection techniques have identified a number of proto-ets homologs in model systems from such diverse evolutionary sources as chicken, man and Drosophila. Using these proto-ets gene probes, we have identified homologous sea urchin sequences. The sequences are strikingly homologous to

that of the virus, sharing more than 266 out of 298 nucleotides analyzed between sea urchin and viral ets-2, and 268 out of 298 for sea urchin and human ets-2, a homology of 89% and 90%, respectively. More significantly, there was a 92% homology of predicted amino acids shared by sea urchin and the chicken viral genomes for ets-2. The homology of predicted amino acids for the ets-2 gene in sea urchin and for human ets-2, surprisingly, was 94%, the highest oncogene homology thus far noted for any species so divergent. Unlike the ets genes in the human genome, the sea urchin gene was without any introns.

The expression of ets-2-related mRNA occurred early during sea urchin embryogenesis. Unlike the human ets-2 gene mRNA expression pattern, the sea urchin mRNA only had a single 5.7 kb message which is transcribed from 5 min to 1,020 min (17 hr) post-fertilization and which reduces to a negligible amount thereafter.

Publications:

Chen, Z.-Q., Ulsh, L. S., DuBois, G. and Shih, T. Y.: Post-translational processing of p21 ras proteins involves palmitylation of the C-terminal tetrapeptide containing cysteine-186. J. Virol. 56: 607-612, 1985.

Shih, T. Y., Clanton, D. J., Hattori, S., Ulsh, L. S. and Chen, Z.-Q.: Structure and function of p21 ras proteins: Biochemical, immunochemical and site-directed mutagenesis studies. In Colburn, N. H., Moses, H. L., Stanbridge, E. J. and Fox, C. F. (Eds.): Growth Factors, Tumor Promoters, and Cancer Genes. New York, Alan R. Liss, Inc. (In Press)

Shih, T. Y., Hattori, S., Clanton, D., Ulsh, L., Chen, Z.-Q., Lautenberger, J. and Papas, T. S.: Structure and function of p21 ras proteins. In Papas, T. S. and Vande Woude, G. F. (Eds.): Gene Amplification and Analysis: Oncogenes. New York, Elsevier, Vol. 4, 1986 (In Press)

Patents:

None

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01CP05485-01 LMO

PERIOD COVERED

October 1, 1985 to September 30, 1986

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Application of Monoclonal Antibodies to the Study of Oncogene Products

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	R. J. Fisher	Expert	LMO	NCI
Others:	S. Fujiwara	Visiting Fellow	LMO	NCI
	N. K. Bhat	Visiting Fellow	LMO	NCI
	T. S. Papas	Chief	LMO	NCI

COOPERATING UNITS (if any)

LBI-Basic Research Program, Frederick, MD (A. Seth)

LAB/BRANCH

Laboratory of Molecular Oncology

SECTION

Carcinogenesis Regulation Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701-1013

TOTAL MAN-YEARS:

1.2

PROFESSIONAL:

1.2

OTHER:

0.0

CHECK APPROPRIATE BOXES)

- (a) Human subjects (b) Human tissues (c) Neither
- (a1) Minors
- (a2) Interviews

SUMMARY OF WORK (Use standard un-reduced type. Do not exceed the space provided.)

c-myc and c-ets (1 and 2) genes are cellular homologues of the oncogenes carried by the avian myelocytomatosis virus MC29 and the avian acute leukemia virus E26, respectively. These genes are suspected to have some roles in the pathogenesis of certain types of human malignancy. Production of monoclonal antibodies against products of these genes was planned for application to the biological and biochemical characterization of these products. A monoclonal antibody reactive with human c-myc proteins has been isolated using a synthetic oligopeptide antigen. This antibody detects an additional polypeptide in addition to c-myc proteins, and its possible relationship to c-myc products is being investigated. In the course of pulse-labeling and subsequent chase of COLO 320 cells, four different polypeptides have been detected by this antibody. They possibly represent different species of human c-myc proteins and precursor-product relationships are suggested between some of these polypeptides. Proteins recognized by this antibody are being purified for amino acid sequencing studies.

PROJECT DESCRIPTIONNames, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

R. J. Fisher	Expert	LMO	NCI
S. Fujiwara	Visiting Fellow	LMO	NCI
N. K. Bhat	Visiting Fellow	LMO	NCI
T. S. Papas	Chief	LMO	NCI

Objectives:

The immediate objective of this project is to produce immunological probes which recognize specific sites on the molecule of proteins encoded by human *c-myc*, *c-ets-1* and *c-ets-2* genes. These probes are applied to biological and biochemical characterization of the gene products in search of their normal functions. In addition, as specific probes for certain regions of protein molecules, they can be utilized to investigate protein structure at individual domain levels. Detailed analysis of the structure of these human oncogene products performed in relation to functional significances should give insights into molecular mechanisms by which these proteins mediate malignant cellular transformation.

Methods Employed:

The antigens used are produced from *c-myc*, *c-ets-1* or *c-ets-2* genes which have been expressed in *E. coli* or from oligopeptides synthesized from the derived amino acid sequences of these genes. BALB/c mice are immunized with these antigens and hybridomas are generated using standard procedures, including polyethylene glycol-mediated cell fusion and hypoxanthine, aminopterin-thymidine (HAT) selection of hybrid cells. Hybridoma culture fluids are screened for specific antibodies by the enzyme-linked immunosorbent assay (ELISA). Native cellular proteins encoded by these genes are detected by monoclonal antibodies using immunoprecipitation and immunoblot techniques. Proteolytic fragment profiles of the detected proteins are obtained by digestion with staphylococcal V8 protease or trypsin, followed by one-dimensional or two-dimensional electrophoresis analysis. The proteins detected by monoclonal antibodies are purified by high performance liquid chromatography (HPLC).

Major Findings:

A monoclonal antibody to a synthetic oligopeptide derived from an amino acid sequence close to the amino terminus of the second human *c-myc* exon was produced and was shown to react with native *c-myc* proteins of various human cell lines. This antibody also recognized a protein expressed by cloned human *c-myc* genes in eukaryotic cells, substantiating its specificity to the *c-myc* locus. In addition to *c-myc* proteins, the antibody recognized another protein (80K) which showed a limited similarity with the *c-myc* proteins in staphylococcal V8 protease partial cleavage mapping. The possible relationship of this protein to the *c-myc* products is being examined further. Four polypeptides generated in the course of pulse-labeling with [³⁵S]-methionine and subsequent chase

of COLO 320 cells were recognized by the monoclonal antibody. They have approximate molecular weights of 58K, 60K, 62K and 66K, and possibly represent different species of proteins encoded by the human c-myc gene. Precursor-product relationships are suggested between some of these polypeptides. The antibody was also utilized in purification of the human c-myc products for amino acid sequencing studies.

Publications:

Ascione, R., Sacchi, N., Watson, D. K., Fisher, R. J., Fujiwara, S., Seth, A. and Papas, T. S.: Oncogenes: Molecular probes for clinical application in malignant diseases. Gene Anal. Tech. 3: 25-39, 1986.

Papas, T. S., Blair, D. G., Fisher, R. J., Watson, D. K., Sacchi, N., Fujiwara, S., Bhat, N. and Ascione, R.: The ets genes. In Reddy, E. P., Curran, T. and Skalka, A. (Eds.): The Oncogene Handbook. New York, Elsevier (In Press)

Patents:

None

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01CP05486-01 LMO

PERIOD COVERED

October 1, 1985 to September 30, 1986

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

cDNA Cloning, Sequencing and Expression of Human ets-1 and ets-2

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, end institute affiliation)

PI: T. S. Papas Chief LMO NCI

Other: V. Rao Visiting Fellow LMO NCI

COOPERATING UNITS (if any)

Nucleic Acid and Protein Synthesis Laboratory, Program Resources, Inc., Frederick, MD (E. S. P. Reddy)

LAB/BRANCH

Laboratory of Molecular Oncology

SECTION

Carcinogenesis Regulation Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701-1013

TOTAL MAN-YEARS:

0.6

PROFESSIONAL:

0.6

OTHER:

0.0

CHECK APPROPRIATE BOX(ES)

- (a) Human subjects (b) Human tissues (c) Neither
- (a1) Minors
- (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The avian erythroblastosis virus, E26, is a replication-defective retrovirus which causes erythroblastosis and myeloblastosis in chickens. The transforming gene of E26 includes elements from two proto-onc genes, chicken proto-myb and chicken proto-ets, and Agag from the viral gag gene. Human genomic clones homologous to the ets region of the E26 virus were molecularly cloned and shown to be closely related to the v-ets region by hybridization and partial sequence analysis. The human ets DNA is located on two different chromosomes. The human ets-1 locus on chromosome 11 encodes a single mRNA of 6.8 kb; the second ets-2 locus on chromosome 21 encodes three mRNAs of 4.7, 3.2 and 2.7 kb. In order to study the structural organization and splicing mechanism of the human ets-1 and ets-2 genes, a cDNA library was prepared from a human COLO 320 cell line which expresses very high levels of ets-specific transcripts. Several recombinant clones reactive with ets-1 and ets-2 probes were isolated. These cDNA clones are being characterized by restriction mapping and Southern blot analysis into a different family of mRNAs. These clones are also being sequenced. There is preliminary evidence to state that these multiple transcripts, in the case of ets-1 and ets-2, may have been generated through alternative splicing events. The full length cDNA clones of ets-1 and ets-2 are being expressed in vitro and in vivo in *E. coli* and in mammalian cells. With the above study, it may become possible to define precisely any small differences between the viral genes and their cellular counterparts--differences that may be crucial for the regulation of the "genes" expression or the range of their biological functions. These regulatory genes, which have been conserved during evolution, may have some role in cell differentiation and multiplication.

PROJECT DESCRIPTIONNames, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

T. S. Papas	Chief	LMO	NCI
V. Rao	Visiting Fellow	LMO	NCI

Objectives:

To study the structural organization and biological functions of the human ets-1 and ets-2 genes by preparing a cDNA library from COLO cells and isolating ets-1 and ets-2 cDNA clones. To characterize these cDNA clones by restriction mapping and nucleotide sequencing. Finally, to express the full-length clones in prokaryotic and eukaryotic vector systems.

Methods Employed:

A cDNA library representing the mRNA population is constructed using polyadenylated RNA extracted from COLO 320 cells. This RNA is reverse transcribed using AMV reverse transcriptase; the second strand synthesis is catalyzed by *E. coli* DNA polI. Digestion with S1 nuclease removes the single-stranded hairpin region connecting the two strands. The double-stranded cDNAs are treated with EcoRI methylase and EcoRI linkers are ligated to the ends. The double-stranded cDNA is size fractionated and ligated to λ or plasmid vectors.

The ets-1- and ets-2-specific cDNA clones are identified by screening the library with ets-1 and ets-2 probes using the filter hybridization method. These cDNA clones are further subcloned and characterized by restriction mapping and Southern blotting techniques. Northern blot analysis is also performed using these cDNA clones as probes. These clones are sequenced by the chemical modification and cleavage method of Maxam and Gilbert, and also the dideoxy chain termination method of Sanger by cloning into M13 mp vectors using the following sequencing strategies of shotgun, forced, random and nonrandom cloning. The full-length cDNA clones of ets-1 and ets-2 will then be cloned into prokaryotic and eukaryotic expression vectors. The transcripts will be analyzed by Northern technique and the protein product by gel electrophoresis, immunoprecipitation, Western blotting, immunofluorescence and amino acid analysis.

Major Findings:

1. A cDNA library was prepared from COLO 320 cells. Five nearly full-length cDNA clones reactive with the human ets-1 genomic probe were isolated.
2. Five recombinant clones specific to human ets-2 cDNA probes have also been identified.
3. Two of the five ets-1 cDNA clones were derived from the same class of mRNA as determined by size and restriction map analysis. The above finding suggests for the first time the existence of multiple transcripts of the ets-1 gene, which may be due to an alternative splicing mechanism.

4. The human ets-2 cDNA clones analyzed so far fell roughly into two categories of mRNA based on size and restriction map analysis. Each of these share a common 5' region. This data has been obtained by Southern blot analysis and partial sequence. There is also preliminary evidence for an alternative splicing event.

5. Further work is in progress related to nucleotide sequencing, characterization of other clones and studying expression in E. coli and mammalian cells.

Publications:

None

Patents:

None

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01CP05493-01 LMO

PERIOD COVERED

October 1, 1985 to September 30, 1986

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Expression of HTLV-III Envelope Gene in Eukaryotic (SV40)-Based Expression Vector

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: T. S. Papas Chief LMO NCI

Other: V. Rao Visiting Fellow LMO NCI

COOPERATING UNITS (if any)

Nucleic Acid and Protein Synthesis Laboratory, Program Resources, Inc., Frederick, MD (E. S. P. Reddy)

LAB/BRANCH

Laboratory of Molecular Oncology

SECTION

Carcinogenesis Regulation Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701-1013

TOTAL MAN-YEARS:

0.6

PROFESSIONAL:

0.6

OTHER:

0.0

CHECK APPROPRIATE BOX(ES)

- (a) Human subjects (b) Human tissues (c) Neither
- (a1) Minors
- (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Several constructs using the power of recombinant DNA technology were employed to express in eukaryotic vector systems the envelope genes encoded by the HTLV-III virus. One type of construct incorporates a portion of the HTLV-III envelope gene fused to an SV40 promoter region, complete with splice and polyadenylation signals. A second type of expression vector construct fused the HTLV-III viral envelope gene with the SV40 T antigen amino terminal region, which can elicit proteins recognized by specific antibodies directly against T antigen. Both types of constructs, after cotransfection into TK- cells along with the thymidine kinase gene, gave rise to selective transformants that enabled permanent lines to be established. In particular, 8 out of 10 such cell lines were found to be expressing HTLV-III envelope specific mRNA. Four of these cell lines were expressing high levels of message, while the other four were moderately expressive. Recombinant vectors containing metallothionein promoter inducible HTLV-III envelope genes were recently constructed containing SV40 T antigen splice and polyadenylation signals and are being introduced into embryonic mice for transgenic expression studies.

PROJECT DESCRIPTIONNames, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

T. S. Papas	Chief	LMO	NCI
V. Rao	Visiting Fellow	LMO	NCI

Objectives:

To clone the entire envelope gene of the HTLV-III virus and stably express the product in a eukaryotic cellular system appropriately modified to be recognized by viral-specific antisera.

Methods Employed:

A clone of the HTLV-III envelope gene containing fragment was ligated with the appropriate promoter, splice acceptor/donor and polyadenylation signal into SV40 vectors constructed in this Laboratory. Cells are cotransfected by the calcium precipitation technique using the TK selectable gene as a marker in mouse embryonic cells (LTK⁻) negative for thymidine kinase. HAT selection enables the detection of successful transformants, a portion of which is transiently transfected into HeLa cells to facilitate the screening of genes for maximal expression of HTLV-III mRNA utilizing Southern and Northern transfer analyses. Stable transformants were maintained and subjected to Northern blot analytic techniques for mRNA production, and Western blotting and immunoprecipitation analysis for protein detection.

Major Findings:

Two different constructs were made in order to express a portion of and the complete the envelope gene. In the first construct, a portion of the envelope gene was fused with SV40 T antigen (10-11 amino acids near the amino terminal region). In the second construct the complete envelope gene was placed under the SV40 promoter with the splice and polyadenylation signals. Both of the above constructs were transfected into LTK⁻ cells (mouse embryo fibroblasts), along with the thymidine kinase gene, and permanent lines were established. Ten lines were screened for expression at the level of transcripts. RNA was isolated from these cell lines, Northern blotted and probed with the HTLV-III envelope gene probe. Four lines were expressing high levels of transcripts and four others, moderate levels. The above eight lines were checked for the expression of the envelope gene at the level of protein. To our surprise, none of these lines expressed detectable levels of protein, although high levels of RNA were produced.

Recent results suggest that expression of some genes of HTLV-III (art and tat) are needed for the efficient expression of the HTLV-III envelope gene. We are planning to transfect art genes (under SV40 promoter) into the permanent cell lines (which are expressing HTLV-III envelope transcripts) and check for HTLV-III envelope protein.

We have constructed the envelope gene of HTLV-III under the control of metallothionein promoter. Such a construct will be introduced into transgenic mice.

Publications:

None

Patents:

None

ANNUAL REPORT OF

LABORATORY OF MOLECULAR VIROLOGY
BIOLOGICAL CARCINOGENESIS PROGRAM
DIVISION OF CANCER ETIOLOGY
NATIONAL CANCER INSTITUTE

October 1, 1985 through September 30, 1986

The Laboratory of Molecular Virology (1) analyzes the mechanism of gene expression in normal and transformed eukaryotic cells; (2) uses viruses as tools for probing cellular regulatory mechanisms; (3) develops and applies biological, biochemical and immunological procedures to obtain evidence for the mechanism by which antigens are recognized by the immune system; (4) plans and conducts research on transforming proteins to define their properties in normal cells and their potential role in the development of neoplasms; and (5) investigates the mechanisms by which cellular gene expression, viral gene expression, and interactions between viruses may influence the transformation of cells.

The Virus Tumor Biology Section (1) characterizes the nucleotide sequence from regions of viral and cellular DNA thought to be involved in gene expression; (2) investigates the properties of cellular and viral transforming genes and their protein products; (3) evaluates the mechanisms by which viral and cellular proteins affect the level of gene expression; and (4) develops eukaryotic viral vectors to study gene expression.

The Cell Physiology Section (1) investigates the molecular elements essential for cellular transformation; (2) studies the properties of cell surface molecules in expression of the cellular phenotype; (3) uses recombinant DNA techniques and molecular genetics to study the elements involved in gene regulation; and (4) employs prokaryotic host-vector-systems to examine sequences involved in efficient gene expression and protein production.

The elucidation of signals associated with gene expression is among the primary objectives of the Laboratory of Molecular Virology. In particular, our interest has been directed toward regulatory events which take place at the level of transcription and processing of RNA. We have been involved in the elucidation and analysis of novel genetic elements, enhancer sequences, which appear to be responsible for controlling the rate at which particular genes are transcribed. We have demonstrated the existence of these enhancer sequences not only in the genomes of DNA viruses such as SV40, JCV and BKV, but also in the long terminal repeats (LTRs) of retroviruses. Using a combination of in vivo and in vitro assays, we have demonstrated that enhancer sequences often show host-cell specificity, and thus may be among the elements involved in controlling the host range of certain viruses as well as the tissue-specific expression of certain eukaryotic genes. A number of laboratories have now shown that enhancers are critical elements in determining the activity of eukaryotic genes and that they function in a tissue- or organ-specific fashion. A major effort in our laboratory will be directed at determining whether or not enhancer sequences play a role in the developmental and tissue-specific regulation of gene expression. In addition, we are interested in mutagenizing regions of enhancer elements to elucidate

those sets of nucleotides associated with the general activation phenomenon as well as the cellular specificity. In vivo and in vitro experiments have been designed in an attempt to examine the mechanism by which the activator/enhancer sequences function. Currently, we have embarked on a number of experiments to define and characterize the biological macromolecules which interact with these regulatory elements.

A particularly fruitful avenue of investigation has involved the development of transgenic mice. Using JC virus as a model system, we have been able to establish lines of mice with specific neurologic defects including a dysmyelination syndrome and tumors of the adrenal medulla. Animals have also been established which carry "foreign" class I histocompatibility antigens (see below). These will be important in studying a number of basic principles associated with immunosurveillance and the distinction between foreign and self.

An understanding of the structure and function of the class I histocompatibility antigens (the classical transplantation antigens) and in particular, the roles of these cell-surface antigens in relation to the neoplastic state has been a subject of considerable interest. These studies are of singular importance because the ability of the immune system to identify and destroy tumor cells depends upon their presentation by the class I antigens to the cytotoxic T-lymphocytes. A major goal is to obtain an understanding of the factors which govern immune recognition of foreign cells. Attempts are directed at in vivo and in vitro immune modulation which will hopefully enhance the ability of the host to recognize tumor cells as "foreign" and to eliminate them by immunologic means.

Molecular cloning and identification of class I loci has led to the finding of a gene that encodes a soluble or secreted class I-related antigen. Because of variations in the level of expression of this gene in different inbred mouse strains and an unusual tissue-restriction in its expression, it was suggested that this soluble histocompatibility antigen represented as a serum protein, perhaps a tolerogenic form of the class I antigens, which could act as a blocking factor to regulate the function of cytotoxic T-cells in the process of immune surveillance. Studies are in progress to test this hypothesis by using the secreted class I antigen to modulate T-lymphocyte recognition of tumor cells.

A considerable effort has been directed toward the study of mammalian oncogenes and their counterparts in yeast. It is hoped that this information will contribute to an understanding of the process of cell transformation and tumorigenesis. Studies in progress are designed to investigate the role of oncogenes in inducing cell transformation as well as the cellular factors which contribute to metastases in animal models.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01CP05101-08 LMV

PERIOD COVERED

October 1, 1985 through September 30, 1986

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Studies on the Molecular Mechanisms for Malignant Transformation of Cells

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	Gilbert Jay	Chief, Cell Physiology Section	LMV	NCI
Others:	Stephen Hinrichs	Medical Staff Fellow	LMV	NCI
	Michael Nerenberg	Medical Staff Fellow	LMV	NCI
	George Khoury	Chief	LMV	NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Molecular Virology

SECTION

Cell Physiology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

1.4

PROFESSIONAL:

1.4

OTHER:

0

CHECK APPROPRIATE BOX(ES)

- (a) Human subjects
 (b) Human tissues
 (c) Neither
 (a1) Minors
 (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The goal of this project is to investigate the molecular mechanisms underlying the malignant transformation of cells by the human T-lymphotropic virus type I (HTLV-I). We have defined the basis for the derivation of the "transforming" gene of HTLV-I and have analyzed its mode of action in transgenic mice.

PROJECT DESCRIPTIONNames, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Gilbert Jay	Chief, Cell Physiology Section	LMV	NCI
Steven Hinrichs	Medical Staff Fellow	LMV	NCI
Michael Nerenberg	Medical Staff Fellow	LMV	NCI
George Khoury	Chief	LMV	NCI

Objectives:

To understand transformation by the tumor virus HTLV-I.

Methods Employed:

Proteins were detected by immunoprecipitation using either conventional or monoclonal antibodies.

Major Findings:

HTLV-I encodes a novel gene product (p40^X which can act to transactivate viral and cellular gene expression. The function of this viral protein is suspected to be responsible for the etiology of adult T-cell leukemias.

1. Using viral DNA as a probe, we have identified and cloned a cellular homologue of the viral p40^X gene. This finding suggests that the viral gene may be derived from the host genome, and that an understanding of the role of the cellular gene may lead to a better understanding of the function of the viral counterpart.
2. To study the role of the HTLV-I p40^X, we have derived transgenic mice which carry the p40^X gene under the control of the authentic viral regulatory elements. The transgenic mice expressing p40^X at high levels in the thymus died shortly after birth. We are now studying the pathology of these mice to determine the basis for their death.

Publications:

Rhim, J. S., Sanford, K. K., Arnstein, P., Fujita, J., Jay, G. and Aaronson, S.: Human epithelia cell carcinogenesis: Combined action of DNA and RNA tumor viruses produces malignant transformation of primary human epidermal keratinocytes. In Barrett, J. C. (Ed.): Carcinogenesis: A Comprehensive Survey. New York, Raven Press, 1985, pp. 57-66.

Patents:

None

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01CP05214-06 LMV

PERIOD COVERED

October 1, 1985 through September 30, 1986

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Genetic Elements Regulating the Initiation of Transcription

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	John Brady	Expert	LMV	NCI
Others:	George Khoury	Chief	LMV	NCI
	Mary Loeken	Guest Researcher	LMV	NCI
	Janet Duvall	Biological Aid	LMV	NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Molecular Virology

SECTION

Virus Tumor Biology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

1.2

PROFESSIONAL:

0.7

OTHER:

0.5

CHECK APPROPRIATE BOX(ES)

- (a) Human subjects (b) Human tissues (c) Neither
- (a1) Minors
- (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

To gain further understanding of the mechanisms by which trans-acting factors interact to recognize transcriptional regulatory sequences, we have examined the ability of simian virus 40 (SV40) T-antigen and adenovirus E1A protein to stimulate the adenovirus E2 promoter. An E2-CAT plasmid was cotransfected into monkey kidney cells with plasmids encoding SV40 T-antigen or E1A. CAT activity and RNA is then assayed to estimate the rate of transcriptional activity directed from the E2 promoter. We have found that either SV40 T-antigen or E1A protein will stimulate the E2 promoter in cultured monkey kidney cells (CV-1 cells). Based on experiments in which concentrations of the E2-CAT plasmid or T or E1A-encoding plasmids were varied, it appears that the mechanism by which T and E1A activate the E2 promoter are different. Furthermore, T-antigen in COS-1 cells, which are derived from CV-1 cells by transformation with SV40 and constitutively express T-antigen, does not stimulate the E2-CAT plasmid. E1A, however, is able to stimulate this plasmid in COS-1 cells. This suggests that there are at least two different mechanisms for activation of the E2 promoter, and only the E1A-mediated mechanism is active in COS-1 cells. This also suggests that the different activities of SV40 T-antigen may be separable, since SV40 replication and late gene expression is observed in COS-1 cells. Using synthetic oligonucleotide promoter sequences, we are currently examining whether there are sequences on the E2 promoter which can distinguish between T-antigen and E1A-mediated stimulation. In addition, stereochemical interactions between proteins binding to individual upstream promoter sequences are under investigation.

PROJECT DESCRIPTIONNames, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

John Brady	Expert	LMV	NCI
George Khoury	Chief	LMV	NCI
Mary Loeken	Guest Researcher	LMV	NCI
Janet Duvall	Biological Aid	LMV	NCI

Objectives:

This project is directed toward an analysis of the genetic elements required for the initiation of transcription. It should provide information about structure and function of eukaryotic promoters.

Methods Employed:

Recombinant DNA techniques; eukaryotic cell transfection; nucleic acid hybridization; electrophoretic immunoblot analysis of protein; gel electrophoresis; construction of transcriptional regulatory deletion and point mutants.

Major Findings:

1. SV40 T-antigen and adenovirus E1A oncogene products will stimulate expression from the adenovirus E2 promoter.
2. The mechanism by which T-antigen and E1A activate expression from the E2 promoter appears to require different transcriptional factors.
3. Different biological functions of the SV40 T-antigen may be separable. T-antigen in a COS-1 cell does not trans-activate the adenovirus E2 promoter. COS-1 T-antigen does trans-activate the SV40 late promoter and can also replicate SV40 DNA.

Publications:

Ishii, S., Kadonaga, J. T., Tjian, R., Brady, J. N., Merlino, G. T. and Pastan, I.: Binding of the Sp1 transcription factor by the human Harvey ras1 proto-oncogene promoter. Science 232: 1410-1413, 1986.

Loeken, M. R., Khalili, K., Khoury, G. and Brady, J.: Evidence that polymerase II transcription requires interaction between proteins binding to control sequences. In Botchan, M., Grodzicker, T. and Sharp, P. A. (Eds.): Cancer Cells Vol. 4 - DNA Tumor Viruses: Control of Gene Expression and Replication. New York, Cold Spring Harbor Laboratory, 1986. (In Press)

Loeken, M. R., Khoury, G. and Brady, J.: Stimulation of the adenovirus E2 promoter by SV40 T-antigen or E1A occurs by different mechanisms. Mol. Cell. Biol. 6: 2020-2026, 1986.

Patents:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05216-06 LMV

PERIOD COVERED

October 1, 1985 through September 30, 1986

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Ras Oncogene Regulation in Yeast

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	Ravi Dhar	Visiting Scientist	LMV	NCI
Others:	Diego Breviario	Visiting Fellow	LMV	NCI
	Richard Koller	Biologist	LMV	NCI

COOPERATING UNITS (if any)

Clinical Hematology Branch, NHLBI (M. Ruta); Virus & Cell Biology Research, Merck Sharp & Dohme Research Laboratories, West Point, PA (D. DeFeo-Jones, E. Scolnick)

LAB/BRANCH

Laboratory of Molecular Virology

SECTION

Virus Tumor Biology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

3.0

PROFESSIONAL:

2.0

OTHER:

1.0

CHECK APPROPRIATE BOX(ES)

- (a) Human subjects (b) Human tissues (c) Neither
 (a1) Minors
 (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Two ras genes, c-ras1 and c-ras2, were isolated from the yeast strain Saccharomyces cerevisiae. Transcriptional analysis of yeast ras genes in different culture conditions suggests that the inability of ras2⁻ mutants to grow in non-fermentable carbon sources results from the regulation of ras1 expression. The amount of ras1 mRNA is significantly repressed in cultures grown on the non-fermentable carbon sources ethanol and acetate. As a result, low ras protein is expressed under these conditions in ras2⁻ mutants. This explains the inability of ras2⁻ cells to grow on nonfermentable carbon sources. An extragenic suppressor of ras2⁻ (sra 6-15), which restores growth on ethanol or acetate, leads to an increase in the amount of ras1 mRNA when grown on nonfermentable carbon sources. The pattern of transcriptional regulation for ras1 is not shared by ras2 indicating a differential control of these homologous yeast genes at the level of gene expression.

Three different promoters for transcription have been identified for ras2 mRNA. Ras2 protein is always synthesized. The ras2 gene is regulated at the level of transcription and not at the level of protein synthesis.

PROJECT DESCRIPTIONNames, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Ravi Dhar	Visiting Scientist	LMV	NCI
Diego Breviario	Visiting Fellow	LMV	NCI
Richard Koller	Biologist	LMV	NCI

Objectives:

Our major objectives are:

1. To study the transcriptional and translational regulation of the two ras genes in yeast and their extragenic suppressors.
2. To study the phenotype of ras mutants and correlate gene regulation with biological function.

Methods Employed:

Recombinant DNA technology; Southern and Northern blot analysis; immunoprecipitation; Western blots.

Major Findings:

We have shown the two ras genes, c-ras1 and c-ras2, are differentially regulated.

1. In the presence of glucose, yeast cells express a large amount of ras1 mRNA and ras protein early in their growth phase.
2. Low levels of ras1 mRNA are made very early when cells are grown in nonfermentable carbon sources like ethanol or acetate. Such regulation provides an explanation for the inability of ras2⁻ cells to grow on nonfermentable carbon sources.
3. The inability of ras2⁻ cells to grow on nonfermentable carbon sources can be reversed by an extragenic suppressor, *sra 6-15*.
4. Ras1 cannot substitute for ras2 protein in sporulation, even when ras1 protein is overproduced.
5. C-ras2 is regulated at the level of transcription resulting in three major RNA 5' ends.
6. C-ras2 is not regulated by different carbon sources.
7. Ras2 protein is always synthesized in the cells, even under conditions where total synthesis is reduced by 100-fold (e.g., during heat shock). The ratio of active protein synthesis to that of ras2 protein is always constant.

8. Ras2 polypeptide is downregulated under conditions of sporulation or nutrient starvation.
9. Sporulation of yeast cells can occur in the absence of ras2 protein in rich media under conditions of starvation, which bypass the need for ras2 protein.
10. Contrary to previous reports, we have shown both ras1 and ras2 proteins have a short half-life of less than two minutes.

Publications:

Breviario, D., Hinnebusch, A., Cannon, J., Tatchell, K. and Dhar, R.: Carbon source regulation of RAS1 expression in Saccharomyces cerevisiae and the phenotypes of ras2⁻ cells. Proc. Natl. Acad. Sci. USA 83: 4152-4156, 1986.

Ruta, M., Wolford, R., Dhar, R., Defeo-Jones, D., Ellis, R. W. and Scolnick, E. M.: Nucleotide sequence of the two rat cellular ras^H genes. Mol. Cell. Biol. 6: 1706-1710, 1986.

Patents:

None

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01CP05217-06 LMV

PERIOD COVERED

October 1, 1985 through September 30, 1986

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Studies on the Regulation of SV40 Gene Expression

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.: John Brady Expert LMV NCI

Others: Kamel Khalili Visiting Fellow LMV NCI

Gilbert Jay Chief, Cell Physiology Section LMV NCI

George Khoury Chief LMV NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Molecular Virology

SECTION

Virus Tumor Biology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

0.7

PROFESSIONAL:

0.7

OTHER:

0

CHECK APPROPRIATE BOX(ES)

 (a) Human subjects (b) Human tissues (c) Neither (a1) Minors (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

1. We have analyzed the transcriptional efficiencies of one downstream and two upstream early simian virus 40 (SV40) transcripts synthesized in vitro. Our studies have demonstrated that the presence of upstream AUG initiation codons decrease the initiation of translation at the early SV40 gene products initiation. Labeling of in vitro translation products with proline and serine in an in vitro translation reaction programmed with capped SV40 RNAs resulted in a synthesis of a small protein of 2.7-Kd. In vivo labeling of SV40 infected CV-1 cells demonstrated the accumulation of a peptide of similar size at late times after infection. The function of this novel protein in the lytic cycle of SV40 is currently under investigation.
2. We have employed mutants in the region encoding the carboxy terminus of T-antigen to further investigate a role of T-antigen in SV40 late gene expression. While these mutants have little effect on the efficiency of viral DNA replication, they significantly decrease the yield of infectious virus particles by 3-4 logs. The level of late viral RNA and capsid protein (VP1, VP2 and VP3) are 5- to 15-fold lower in these mutants in comparison to those in the wild-type. Analysis of another late viral polypeptide, the agnoprotein, has demonstrated a significant decrease (over 100-fold) in its synthesis of the 61-amino acid polypeptide.

PROJECT DESCRIPTIONNames, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Kamel Khalili	Visiting Fellow	LMV	NCI
John Brady	Expert	LMV	NCI
Gilbert Jay	Chief, Cell Physiology Section	LMV	NCI
George Khoury	Chief	LMV	NCI

Objectives:

To study the function of the SV40 gene and gene products.

Methods Employed:

Tissue culture; DNA transfection; radiolabeling of DNA, RNA and protein; DNA cloning; polyacrylamide gel electrophoresis; Southern blotting; Northern blotting and hybridization; detection and analysis of protein by immunoprecipitation and SDS-polyacrylamide gel electrophoresis.

Major Findings:

SV40 provides a unique model system for the study of gene regulation. We have used it to make the following observations:

1. Late in the lytic cycle, the expression of T-antigen is regulated by translational control mechanism in which upstream AUGs impede translation of the message.
2. The 23-amino acid leader peptide is synthesized at late times in vivo from the SV40 early message.
3. Carboxy terminal mutants of SV40 T-antigen severely affect production of late messenger RNA and late gene products.

Publications:

Khalili, K., Khoury, G. and Brady, J.: Spacing between SV40 early transcriptional control sequences is important for regulation of early RNA synthesis and gene expression. Mol. Cell. Biol. (In Press)

Nomura, S., Jay, G. and Khoury, G.: Spontaneous deletion mutants resulting from a frame-shift insertion in the SV40 agnogene. J. Virol. 58: 165-172, 1986.

Patents:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05220-06 LMV

PERIOD COVERED

October 1, 1985 through September 30, 1986

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Studies on the Structure and Function of Cell Surface Antigens

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	Gilbert Jay	Chief, Cell Physiology Section	LMV	NCI
Others:	Jonathan Vogel	Medical Staff Fellow	LMV	NCI
	Roberta Reynolds	Research Microbiologist	LMV	NCI
	George Khoury	Chief	LMV	NCI
	Laurence Rubin	Guest Researcher	LMV	NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Molecular Virology

SECTION

Cell Physiology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

3.4

PROFESSIONAL:

2.4

OTHER:

1.0

CHECK APPROPRIATE BOX(ES)

- (a) Human subjects (b) Human tissues (c) Neither
- (a1) Minors
- (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

1. We have cloned and analyzed cDNA sequences derived from genes which encode the classical transplantation antigens. Our findings have led to a better understanding of the structure and function of these cell surface antigens, particularly with regard to their role in the presentation of tumor and viral antigens to the immune system.
2. We have studied the expression and function of the human interleukin-2 receptor. Our findings suggest the existence of a secreted interleukin-2 receptor which can bind interleukin-2 efficiently. Furthermore, by using DNA-mediated gene transfer, we have demonstrated that the interleukin-2 receptor can function effectively in non-lymphoid cells.

PROJECT DESCRIPTIONNames, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Gilbert Jay	Chief, Cell Physiology Section	LMV	NCI
Jonathan Vogel	Medical Staff Fellow	LMV	NCI
Roberta Reynolds	Research Microbiologist	LMV	NCI
George Khoury	Chief	LMV	NCI
Laurence Rubin	Guest Researcher	LMV	NCI

Objectives:

We wish to study the organization and expression of the genes coding for the H-2 histocompatibility antigens and interleukin-2 by molecular cloning technologies.

Methods Employed:

Recombinant DNA techniques; nucleic acid hybridization; gel electrophoresis; electron microscopy; protein analysis by immunological methods in combination with polyacrylamide gel electrophoresis.

Major Findings:

1. In an attempt to understand how class I genes are regulated, we dissected the L^d gene to identify potential control regions. Using a test vector containing the SV40 early promoter placed upstream of the bacterial chloramphenicol acetyltransferase (CAT) gene, we demonstrated the presence of a transcriptional enhancer within the 5' flanking region. The sequence is functional in both orientations and has been mapped within 350-bp upstream of the L^d transcriptional start site. Treatment of cells with interferon increases the accumulation of class I transcripts. Expression of the CAT gene under the control of the L^d enhancer and promoter also can be up-regulated by interferon. Our study shows that the target sequence required for this enhancement resides, at least in part, within the same 350-bp segment which contains the transcriptional enhancer.
2. The maintenance of T-lymphocytes which are important effectors of immune responses requires interleukin-2 (IL-2). The binding of IL-2 to specific cell-surface receptors (IL-2R) has been shown to be essential to the growth and proliferation of activated lymphocytes. A human IL-2R cDNA sequence, placed under the control of the SV40 transcriptional promoter and enhancer, has been transfected into murine L-cells. Single cell analysis by autoradiography was used to show that fibroblastic L-cells, stably expressing human IL-2R, respond to stimulation with IL-2 by DNA synthesis and proliferation. This response is specifically blocked by the addition of an anti-IL-2R monoclonal antibody, anti-Tac. The induction of DNA synthesis by IL-2 is both rapid and dose dependent. The ability of IL-2 to stimulate these transfected L-cells to proliferate demonstrates that a lymphoid environment is not required for the functional interaction between IL-2 and its receptor, and provides a unique model system for the investigation of the molecular basis for the cellular events mediated by IL-2.

3. The binding of interleukin-2 (IL-2) to its cell surface receptor (IL-2R) has been shown to be critical for the growth and proliferation of activated lymphocytes. In addition to the expression of cell surface IL-2R, we have recently demonstrated that activated lymphocytes also release a soluble form of this molecule after stimulation with mitogen or antigen. We show that this released molecule is a complex glycoprotein, containing both N- and O-linked carbohydrates, but whose peptide backbone differs from the cell surface IL-2R by 10,000 daltons. Moreover, the released IL-2R is capable of efficiently binding IL-2, and the interaction between growth factor and the released receptor does not appear to require any accessory molecules. The potential therefore exists for this soluble molecule to compete with cell surface receptors for this essential growth hormone, thus playing an important role in the regulation of the immune response.

Publications:

Bieberich, C., Scangos, G., Tanaka, K. and Jay, G.: Regulated expression of a murine class I gene in transgenic mice. Mol. Cell. Biol. 6: 1339-1342, 1986.

Davidson, W. F., Kress, M., Khoury, G. and Jay, G.: Comparison of HLA class I gene sequences: Derivation of locus-specific oligonucleotide probes specific for HLA-A, HLA-B and HLA-C genes. J. Biol. Chem. 260: 13414-13423, 1985.

Rubin, L. A., Jay, G. and Nelson, D. L.: The released interleukin-2 receptor binds interleukin-2 efficiently. J. Immunol. (In Press)

Vogel, J., Kress, M., Khoury, G. and Jay, G.: Identification of a regulatory sequence at the 5' flanking region of a MHC class I gene. Mol. Cell. Biol. (In Press)

Patents:

None

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01CP05254-05 LMV

PERIOD COVERED

October 1, 1985 through September 30, 1986

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Regulation of Gene Expression

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Kuan-Teh Jeang Medical Staff Fellow LMV NCI

Others: George Khoury Chief LMV NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Molecular Virology

SECTION

Virus Tumor Biology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

1.4

PROFESSIONAL:

1.4

OTHER:

0

CHECK APPROPRIATE BOXES)

- (a) Human subjects (b) Human tissues (c) Neither
 (a1) Minors
 (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

We are interested in understanding the regulation of enhancer dependent gene expression in vivo. Specifically, there are two major areas of interest: (1) regulation of gene expression in undifferentiated cells and (2) interaction(s) of protein factors that may effect enhancer dependent expression. Our studies have focused on the role of DNA binding proteins in the regulation of gene transcription. In particular, we have examined the properties of nuclear factor I (NFI), a protein that has been shown by others to be important in both eukaryotic DNA replication and transcription. Preliminary results indicate: (1) NFI plays little or no role in activating expression of normally silent genes in undifferentiated cells. (2) The juxtaposition of NFI between enhancer and a downstream promoter does not affect enhancer dependent transcription. (3) When NFI binding sites are placed at a distal 3' position, an increase in enhancer dependent transcription is seen. As an adjunct to elucidating the roles of DNA binding proteins in gene regulation, we are also studying the effects of prokaryotic DNA binding proteins and binding sequences placed in a eukaryotic environment.

PROJECT DESCRIPTIONNames, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Kuan-Teh Jeang	Medical Staff Fellow	LMV	NCI
George Khoury	Chief	LMV	NCI

Objectives:

The goal of this study is to understand the regulation of gene expression in differentiated and undifferentiated cells.

Methods Employed:

Transient assay systems; recombinant DNA technology; RNA analysis by hybridization and nuclease protection.

Major Findings:

1. NFI does not affect expression of genes normally silent in undifferentiated cells.
2. Binding of a eukaryotic DNA binding protein between an enhancer and a downstream promoter does not affect gene expression.

Publications:

Laimins, L., Holmgren-König, M. and Khoury, G.: Transcriptional "silencer" element in rat repetitive sequences associated with the rat insulin 1 gene locus. Proc. Natl. Acad. Sci. USA 83: 3151-3155, 1985.

Patents:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05354-04 LMV

PERIOD COVERED

October 1, 1985 through September 30, 1986

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Studies on the Activated Form of the Human Proto-oncogene, c-Ha-ras

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and Institute affiliation)

PI:	George Khoury	Chief	LMV	NCI
Others:	Rudy Pozzatti	Guest Researcher	LMV	NCI
	Mary McCormick	Guest Researcher	LMV	NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Molecular Virology

SECTION

Virus Tumor Biology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

2.2

PROFESSIONAL:

2.2

OTHER:

0

CHECK APPROPRIATE BOX(ES)

- (a) Human subjects (b) Human tissues (c) Neither
- (a1) Minors
- (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

We have transfected various viral and cellular oncogenes into primary cultures of rat embryo cells and have obtained lines of morphologically transformed cells. Transformation with the ras oncogene alone was observed; however, a 10-fold increase in the transformation frequency was obtained when ras was cotransfected with the adenovirus E1A gene. We have examined cell lines transformed by the ras oncogene alone, and by ras plus E1A and have observed a striking difference in their metastatic potential as assayed in nude mice. Specifically, the ras alone transformants are highly metastatic while the two gene transformants show a very low metastatic potential. Transfection of the serotype 2 E1A gene, but not the serotype 12 E1A gene, into the ras alone transformants results in a substantial reduction (at least 10-fold) in the metastatic potential of these cell lines. Experiments are in progress to investigate the mechanism by which the adenovirus type 2 E1A gene suppresses the metastatic potential of the ras alone transformants. In addition, we have begun a line of experimentation designed to isolate genes that are involved in the metastatic potential.

PROJECT DESCRIPTIONNames, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Rudy Pozzatti	Guest Researcher	LMV	NCI
Mary McCormick	Guest Researcher	LMV	NCI
George Khoury	Chief	LMV	NCI

Objectives:

We have observed that primary rat embryo cells transformed by the ras oncogene are highly metastatic when assayed in nude mice. This study is designed to determine how the adenovirus type 2 E1A gene is able to suppress the metastatic phenotype associated with the ras transformed rat embryo cells. In addition we will attempt to identify genes that are involved in the metastatic phenotype by transfecting DNA from a highly metastatic cell line into a recipient cell line that is tumorigenic but not metastatic in nude mice.

Methods Employed:

Isolation and cloning of specific fragments; construction of recombinant vector molecules; extraction of mRNA; Northern and Southern blotting analysis of RNA and DNA; immunoprecipitation; DNA transfection; tumorigenesis assays in nude mice.

Major Findings:

The c-Ha-ras oncogene isolated from the human bladder carcinoma cell line, T-24, is capable of transforming the established mouse cell line NIH 3T3. Conflicting reports exist in the literature about whether or not the ras oncogene alone is capable of stably transforming primary cells in culture. Two groups report successful transformation of human and rodent primary cells using a viral enhancer element to increase the levels of ras gene expression. Others have found that the ras gene alone is incapable of transforming primary cultures of rodent cells. However, addition of a second so-called cooperating oncogene to transfection mixtures resulted in efficient transformation of primary cells.

We have transfected oncogenes into second passage rat embryo cells and have examined the frequency of morphological transformation that results from using a single gene (ras) versus two "cooperating" genes (ras and the adenovirus E1A gene). Transfection of the ras gene alone resulted in low frequency of transformation (one cell in 10^5). Transfection of two-genes resulted in a 10-fold increase in the transformation frequency (one cell in 10^4). Cell lines were established from the one and two-gene transformants in order to examine their phenotype properties.

The most striking phenotypic difference between the two classes of transformants was observed when tumorigenicity assays were performed. Both one and two-gene transformants formed rapidly growing tumors in nude mice when injected subcutaneously. However, seven of the eight ras alone transformants formed

metastases in the lungs of animals bearing subcutaneous tumors. None of six two-gene transformants formed metastases after subcutaneous injection. When the metastatic potential of transformed cell lines was analyzed by intravenous injection of cells, all eight ras alone transformants formed large numbers (> 200) of metastatic nodules in the lungs of IV-injected animals. In contrast the two gene transformants showed very low numbers of lung nodules (5) after intravenous injection.

We have tested the hypothesis that the adenovirus type 2 E1A gene may be able to suppress the metastatic phenotype of ras alone transformed cells by transfecting E1A into four independent ras alone transformed cell lines. Three of the four cell lines showed a substantial reduction (at least 10-fold) in metastatic potential as a result of expression of the type 2 E1A gene. We have obtained preliminary evidence that the adenovirus serotype 12 E1A gene is not capable of suppressing the metastatic potential of ras alone transformed cells. We will pursue these observations by examining the sensitivity of the various types of transformants to lysis in vitro by natural killer cells obtained from nude mice.

In a separate series of experiments, we will attempt to clone gene(s) responsible for the metastatic phenotype. We have made a complete genomic cosmid library from a ras transfected metastatic cell line. DNA from this library will be transfected into a C127/ras cell line that we have shown is tumorigenic but non-metastatic in nude mice. Clones which confer the metastatic phenotype will be isolated from the genome of C127 ras transfectants that have acquired the metastatic phenotype. Cosmid clones that are capable of converting a benign tumorigenic cell (C127/ras) into a fully malignant cell will be characterized by DNA sequence analysis.

Publications:

Pozzatti, R., Muschel, R., Williams, J., Padmanabhan, R., Howard, B., Liotta, L. and Khoury, G.: Primary rat embryo cells transformed by one or two oncogenes show different metastatic potentials. Science 232: 223-227, 1986.

Patents:

None

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01CP05355-04 LMV

PERIOD COVERED

October 1, 1985 through September 30, 1986

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Regulation of Immune Surveillance Against Tumor Cells

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	Gilbert Jay	Chief, Cell Physiology Section	LMV	NCI
Others:	Masato Tanaka	Visiting Fellow	LMV	NCI
	George Khoury	Chief	LMV	NCI

COOPERATING UNITS (If any)

None

LAB/BRANCH

Laboratory of Molecular Virology

SECTION

Cell Physiology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

1.2

PROFESSIONAL:

1.2

OTHER:

0

CHECK APPROPRIATE BOX(ES)

- (a) Human subjects (b) Human tissues (c) Neither
- (a1) Minors
- (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Since the class I molecules are self antigens present on the surface of all cells in the body, the immune system must be rendered tolerant to them. Yet, these class I antigens must be recognized by cytotoxic T-cells in the associative recognition of virus-infected and tumor cells. In our analysis of class I genes, we have identified a related gene which may function to regulate this self-nonsel self recognition. This class I gene is expressed only in the liver and encodes a secreted class I antigen. Our demonstration of the secretion of a class I antigen by the liver has explained a previous observation that liver grafts across histocompatibility barriers were never rejected and has led us to suggest that this molecule serves to modulate class I restriction. We reasoned that a molecule with class I specificity that is constantly secreted into the circulation could act as a "blocking" factor, leading to suppression of class I recognition. The level of expression of such a blocking factor may act directly to modulate self-nonsel self recognition that will destroy aberrant cell types but not normal cells. This hypothesis has significant implications and suggests a means to modulate the host's response to neoplastic and autoimmune diseases. Attempts are being made to determine what regulates the expression of this particular class I gene.

PROJECT DESCRIPTIONNames, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Gilbert Jay	Chief, Cell Physiology Section	LMV	NCI
Masato Tanaka	Visiting Fellow	LMV	NCI
George Khoury	Chief	LMV	NCI

Objectives:

To study the mechanisms involved in regulating immune surveillance.

Methods Employed:

Recombinant DNA techniques; nucleic acid hybridization; gel electrophoresis; electron microscopy; protein analysis by immunological methods in combination with polyacrylamide gel electrophoresis.

Major Findings:

1. The H-2 class I genes encode cell-surface glycoproteins which play a critical role in the immune presentation of aberrant cells. The Q10 class I gene, however, encodes a secreted glycoprotein which is highly homologous to the membrane-bound molecules. While the H-2 genes are activated in all tissue-types, the expression of the Q10 gene is restricted to only the liver. Analysis of DNA from different tissues revealed a unique methylation profile for the Q10 gene in liver. Developmental activation of this gene in newborn mice is also reflected by a coordinated temporal change in DNA methylation. By comparing the methylation profiles between congenic mice which differed in their levels of expression of the Q10 gene, it is observed that methylation at the 3' flanking region correlates with expression. Methylations were at both GC and CC sequences. Since treatment of newborns with 5-azacytidine which led to inhibition of methylation resulted in the suppression of Q10, we conclude that hypermethylation in the 3' flanking region is responsible, at least in part, for the activation of the Q10 gene in the liver.
2. We have examined the mid-gestation mouse embryo for transcripts related to the secreted transplantation antigen Q10 and show that this gene is transcribed in the endoderm of the visceral yolk sac (VYS). Its level of expression is highest at day 14, and then declines as development proceeds. Concurrently with the decrease in yolk sac expression, the amount of transcripts accumulating in the fetal liver increases during late embryogenesis.

Publications:

Stein, P., Barra, Y., Jay, G. and Strickland, S.: Expression of a secreted transplantation antigen gene during murine embryogenesis. Mol. Cell. Biol. (In Press)

Tanaka, K., Barra, Y., Isselbacher, K. J., Khoury, G. and Jay, G.: Developmental and tissue-specific regulation of the Q10 class I gene by DNA methylation. Proc. Natl. Acad. Sci. USA (In Press)

Patents:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
 NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05390-03 LMV

PERIOD COVERED

October 1, 1985 through September 30, 1986

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

How Do Tumor Cells Escape Immune Surveillance?

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Gilbert Jay Chief, Cell Physiology Section LMV NCI
 Others: Kenichi Tanaka Visiting Associate LMV NCI
 Hiroaki Hayashi Visiting Fellow LMV NCI
 George Khoury Chief LMV NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Molecular Virology

SECTION

Cell Physiology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

2.2

PROFESSIONAL:

2.2

OTHER:

0

CHECK APPROPRIATE BOX(ES)

- (a) Human subjects (b) Human tissues (c) Neither
 (a1) Minors
 (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The classical transplantation antigens (the major histocompatibility complex class I antigens) play a key role in host defense against cells expressing foreign antigens. Several naturally occurring tumors and virally transformed cells show an overall suppression of these surface antigens. Since the class I molecules are required in the presentation of neoantigens on tumor cells to the cytotoxic T-lymphocytes, their absence from the cell surface may lead to the escape of these tumors from immunosurveillance. To test this possibility, a functional class I gene was transfected into human adenovirus 12-transformed mouse cells which do not express detectable levels of class I antigens; the transformants were tested for expression of the transfected gene and for changes in oncogenicity. The expression of a single class I gene, introduced by DNA-mediated gene transfer into highly tumorigenic adenovirus 12-transformed cells, was sufficient to abrogate the oncogenicity of these cells. Treatment of adenovirus 12-transformed cells with interferon led to derepression of the endogenous class I genes. Rejection of human adenovirus (Ad12) tumors was observed with intramuscular injections of interferon. Interestingly, Ad12 tumor cells treated with interferon can immunize mice against untreated Ad12 tumors.

PROJECT DESCRIPTIONNames, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Gilbert Jay	Chief, Cell Physiology Section	LMV	NCI
Kenichi Tanaka	Visiting Associate	LMV	NCI
Hiroaki Hayashi	Visiting Fellow	LMV	NCI
George Khoury	Chief	LMV	NCI

Objectives:

Molecular analysis of the escape by tumor cells from immune surveillance.

Methods Employed:

Recombinant DNA techniques; nucleic acid hybridization; gel electrophoresis; electron microscopy; protein analysis by immunological methods in combination with polyacrylamide gel electrophoresis.

Major Findings:

The immune system is involved not only in defense against infections but also against "spontaneously derived" aberrant cells. This latter immune function appears to be essential for the removal of autonomous cell variants that presumably arise frequently in all multicellular organisms. The development of malignant tumors, therefore, represents not only neoplastic transformation, but the failure of host resistance to eliminate certain abnormal cells. Transformation of a cell is insufficient to ensure its escape from immune surveillance. Cells transformed in culture very often do not induce tumors when transplanted back into immunocompetent syngeneic hosts. It is those properties of certain tumor cells allowing them to resist immune recognition which are ultimately responsible for their tumorigenicity.

The major histocompatibility complex class I (H-2) antigens (designated K, D and L in mice) are indispensable for the presentation of cells bearing "foreign" antigens to the cytotoxic T-lymphocytes. The finding that certain malignant tumors, including teratocarcinomas, eccrine porocarcinomas and cervical carcinomas, have markedly reduced or nondetected levels of cell-surface class I antigens (in contrast to their normal cellular counterparts) suggests a possible mechanism for their escape from immune surveillance. In support of this hypothesis is the recent finding that cells transformed by the highly oncogenic strain of human adenovirus (Ad12), in contrast to the nononcogenic strain (Ad5), also express reduced levels of class I antigens on their surfaces. This observation with Ad12 provides an experimental system for demonstrating that the absent or reduced expression of class I antigens is directly responsible for oncogenicity.

We have shown that transfection of a functional class I gene into a highly tumorigenic Ad12-transformed cell line that expresses no detectable class I surface antigens resulted in its complete loss of oncogenicity. Since interferon can induce the expression of class I genes, treatment of mice bearing Ad12 tumors with interferon led to total suppression of tumorigenicity. These findings indicate one possible mechanism for the escape of certain tumors from immune surveillance and suggests future therapeutic approaches for the reversal of certain malignancies.

Publications:

Hayashi, H., Tanaka, K., Jay, F., Khoury, G. and Jay, G.: Modulation of the tumorigenicity of human adenovirus 12 transformed cells by interferon. Cell 43: 263-267, 1985.

Tanaka, K., Hayashi, H., Hamada, C., Khoury, G. and Jay, G.: Expression of MHC class I antigens as a strategy for the potentiation of immune recognition of tumor cells. Proc. Natl. Acad. Sci. USA (In Press)

Patents:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05391-03 LMV

PERIOD COVERED

October 1, 1985 through September 30, 1986

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Transcription Analysis of the SV40 Early and Late Promoter

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	John Brady	Expert	LMV	NCI
Others:	Janet Duvall	Biological Aid	LMV	NCI
	Kamel Khalili	Visiting Fellow	LMV	NCI
	Jeffrey Green	Medical Staff Fellow	LMV	NCI
	George Khoury	Chief	LMV	NCI

COOPERATING UNITS (if any)

Laboratory of Biology of Viruses, NIAID (H. Mishoe)

LAB/BRANCH

Laboratory of Molecular Virology

SECTION

Virus Tumor Biology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

2.0

PROFESSIONAL:

1.5

OTHER:

0.5

CHECK APPROPRIATE BOX(ES)

- (a) Human subjects (b) Human tissues (c) Neither
- (a1) Minors
- (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

We have analyzed the effect of insertion mutants between the SV40 21-base pair (bp) repeats and the early-early (EE) TATA sequence. Insertion of 4-, 42- or 90-bp of DNA at the SV40 NcoI site (m.p. 37) have been analyzed for their effect on SV40 early expression and positioning of the RNA 5' ends. Insertion of 4-bp reduced SV40 early promoter dependent CAT expression by 6- to 8-fold. Increasing the size of the insertion to 42- or 90-bp resulted in a further drop in early gene expression to basal levels. At the RNA level, the 4-bp insertion reduced EE RNA synthesis approximately 10-fold. No concomitant increase in late-early (LE) RNA synthesis was observed. Insertion of 42- or 90-bp of DNA resulted in a decrease in EE RNA synthesis and a stimulation of LE RNA synthesis. Deletion of the SV40 72-bp repeats from the insertion mutants demonstrated that some, but not all, of the LE RNA depends upon the presence of these sequences.

These studies suggest that the ability of RNA polymerase II to utilize the EE (TATA-directed) transcriptional control sequence requires an interaction with the upstream 21-bp repeats and/or the 72-bp repeats. The fact that LE RNA levels in pJ11-in42 CAT and pJ11-in90 CAT were equivalent to the level of EE RNA in pJ11-CAT, yet the level of CAT gene expression was decreased greater than 10-fold, suggests that LE mRNA is under translational control.

PROJECT DESCRIPTIONName, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

John Brady	Expert	LMV	NCI
Janet Duvall	Biological Aid	LMV	NCI
Kamel Khalili	Visiting Fellow	LMV	NCI
Jeffrey Green	Medical Staff Fellow	LMV	NCI
George Khoury	Chief	LMV	NCI

Objectives:

To identify transcription regulatory sequences of eukaryotic polymerase II promoters.

Methods Employed:

Recombinant DNA techniques; construction of deletion and point mutants; *in vitro* transcription; electrophoretic analysis of RNA transcripts; nucleic acid hybridization; DNA sequencing; DNA transfection.

Major Findings:

1. The SV40 21-bp repeat, independent of other transcriptional control elements, effectively binds transcriptional factors required for early and late transcription.
2. The 21-bp repeats can stimulate transcription from a heterologous adenovirus-2 major late promoter.
3. In the absence of contiguous transcriptional control sequences, the 21-bp repeats are capable of initiating bidirectional transcription from proximally located sequences.
4. The SV40 early G-H sequence and/or associated transcriptional factors must physically interact with the upstream 21-bp repeats and/or 72-bp repeats in order to function efficiently.
5. The SV40 21-bp repeats are a major regulatory sequence in specifying the site of early RNA initiation.
6. SV40 late-early RNA is translated inefficiently.

Publications:

Khalili, K., Khoury, G. and Brady, J.: Spacing between SV40 early transcriptional control sequences is important for regulation of early RNA synthesis and gene expression. Mol. Cell. Biol. (In Press)

Patents:

None

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01CP05392-03 LMV

PERIOD COVERED

October 1, 1985 through September 30, 1986

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Regulation of SV40 Late Transcription by Large T-Antigen

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	John Brady	Expert	LMV	NCI
Others:	George Khoury	Chief	LMV	NCI
	Mary Loeken	Guest Researcher	LMV	NCI
	Mary Ann Thompson	Staff Fellow	LMV	NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Molecular Virology

SECTION

Virus Tumor Biology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

1.7

PROFESSIONAL:

1.7

OTHER:

0

CHECK APPROPRIATE BOX(ES)

- (a) Human subjects (b) Human tissues (c) Neither
- (a1) Minors
- (a2) Interviews

SUMMARY OF WORK (Use standard unexpanded type. Do not exceed the space provided.)

In previous experiments, we have demonstrated that the SV40 late promoter could be trans-activated by simian virus 40 (SV40) T-antigen in the absence of DNA replication. Activation could be achieved by either cotransfection of the late promoter with a plasmid coding for T-antigen or by transfection of the late promoter into COS-1 cells which constitutively express T-antigen. In the latter case, it was not clear whether expression of the endogenous T-antigen was continuously required or whether a set of cellular transcription factors alone led to the activation of the late promoter. To test these alternatives, we have performed transfection experiments in ts2 COS cells, which express the ts 1609 SV40 T-antigen. Transfection at the non-permissive temperature (40°C) resulted in 5- to 10-fold reduction in SV40 late promoter activity compared to the permissive temperature (32°C).

An *in vitro* transcription system has been developed in order to study the mechanism of late promoter activation. Manley whole cell extracts were prepared from the trans-activation-positive COS-1 cell line. Preincubation of an SV40 DNA template with the COS-1 extract preferentially increases transcription from the major late start site at m.p. 325. Activation of the late promoter required optimization of the DNA template concentration ratio of COS-1 and HeLa extracts, and the length and temperature of preincubation. Under these conditions, increased transcription from the other SV40 late initiation sites or from the adeno major late promoter was not observed. The promoter sequence requirements for activation of the SV40 late promoter and the requirement for SV40 T-antigen in the preincubation COS-1 extract are presently being analyzed.

PROJECT DESCRIPTIONNames, Title, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

John Brady	Expert	LMV	NCI
George Khoury	Chief	LMV	NCI
Mary Loeken	Guest Researcher	LMV	NCI
Mary Ann Thompson	Staff Fellow	LMV	NCI

Objectives:

Identify mechanisms by which eukaryotic genes, transcribed by RNA polymerase II, are regulated at the transcriptional level.

Methods Employed:

Recombinant DNA techniques; eukaryotic cell transfection; nucleic acid hybridization; electrophoretic immunoblot analysis of protein; gel electrophoresis; construction of deletion and point mutants; in vivo competition assays.

Major Findings:

1. SV40 large T-antigen stimulates transcription from the SV40 late promoter.
2. Analysis of promoter mutants and in vivo competition experiments indicate that binding of T-antigen to sites I and II and the 72-bp repeats are important for efficient induction of late transcription.
3. T-antigen activation of the SV40 late promoter may mediate removal of negative transcriptional factors.
4. In vivo competition studies suggest that efficient binding of trans-acting factors requires the presence, in cis, of at least two SV40 regulatory domains. The spacing between the sequences is critical, suggesting a cooperative interaction.
5. Using temperature sensitive (ts) mutants of SV40 T-antigen, we have determined that T-antigen function is required continuously for activation of the SV40 late promoter. These results suggest that T-antigen is directly required for activation, either through interaction with the DNA template or modification/activation of cellular transcription factors.
6. An in vitro transcription system has been developed in which we observe stimulation of SV40 late transcription. This system is critical for the isolation of transcription factors and analysis of transcription at the molecular level.

Publications:

Brady, J., Feigenbaum, L. and Khoury, G.: Viral enhancer elements. In Notkins, A. L. and Oldstone, M. B. A. (Eds.): Concepts in Viral Pathogenesis. New York, Springer-Verlag (In Press)

Brady, J., Loeken, M. R. and Khoury, G.: Interaction between two transcriptional control sequences required for tumor-antigen-mediated simian virus 40 late gene expression. Proc. Natl. Acad. Sci. USA 82: 7299-7303, 1985.

Khoury, G., Khalili, K., Duvall, J. and Brady, J.: The role of cis- and trans-acting functions in SV40 gene regulation. In Crooke, S. T. and Poste, G. (Eds.): New Frontiers in the Study of Gene Functions (In Press)

Patents:

None

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01CP05393-03 LMV

PERIOD COVERED

October 1, 1985 through September 30, 1986

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Effects of JC Virus Early Region in Transgenic Mice

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	George Khoury	Chief	LMV	NCI
Others:	Judy Small	Guest Researcher	LMV	NCI
	Lionel Feigenbaum	Microbiologist	LMV	NCI
	Jeffrey Green	Medical Staff Fellow	LMV	NCI

COOPERATING UNITS (if any)

Department of Biology, The Johns Hopkins University, Baltimore, MD (G. Scangos)

LAB/BRANCH

Laboratory of Molecular Virology

SECTION

Virus Tumor Biology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

3.0

PROFESSIONAL:

3.0

OTHER:

0

CHECK APPROPRIATE BOXES)

- (a) Human subjects (b) Human tissues (c) Neither
 (a1) Minors
 (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

JC virus is a human papovavirus implicated as the causative agent in the demyelinating disease progressive multifocal leukoencephalopathy (PML). JCV has also been associated with tumors in some PML patients. It is the intent of this study to determine if introduction of JCV into transgenic mice would provide an animal model to study these human diseases. Two distinct disease patterns were observed. Three lines of mice developed a neurological disorder, in which myelin production was diminished or absent in the central nervous system. This provides a model to study the demyelinating disease PML. Four transgenic mice developed tumors, diagnosed as neuroblastomas of the adrenal medulla. Production of these mice may provide an insight into and how tumors are induced by the JC virus and how analogous tumors arise in humans.

PROJECT DESCRIPTIONNames, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

George Khoury	Chief	LMV	NCI
Judy Small	Guest Researcher	LMV	NCI
Lionel Feigenbaum	Microbiologist	LMV	NCI
Jeffrey Green	Medical Staff Fellow	LMV	NCI

Objectives:

To study the biology of JC virus effects of JCV using transgenic mice as an animal model.

Methods Employed:

Mouse embryo injection; embryo transfer to pseudopregnant female mice; Southern and Northern hybridizations; immunofluorescence; immunoprecipitation; in situ hybridizations.

Major Findings:

1. JC virus is capable of expression when inserted into the mouse genome and is apparently regulated in a tissue-specific manner.
2. Neurological disease results from expression of JC virus in the brain of transgenic mice, specifically in the oligodendroglia, or the myelin-producing cells. This causes a dysmyelination in the central nervous system.
3. Tumors are induced in the transgenic mice (four of five), and are highly specific to the adrenal medulla. It is possible that a pregnancy-induced immunosuppression induced development of tumors in the three female transgenic mice.

Publications:

Feigenbaum, L. and Khoury, G.: The role of enhancer elements in viral host range and pathogenicity. In Fields, B., Martin, M. A. and Kamely, D. (Eds.): Genetically Altered Viruses and the Environment. New York, Cold Spring Harbor Laboratory, 1985, pp. 181-194.

Small, J. A., Scangos, G. A., Cork, L., Jay, G. and Khoury, G.: The early region of human papovavirus JC induces dysmyelination in transgenic mice. Cell (In Press)

Patents:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05394-03 LMV

PERIOD COVERED

October 1, 1985 through September 30, 1986

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Enhancer Elements in B-lymphocytes and T-lymphocytes

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	George Khoury	Chief	LMV	NCI
Others:	John Brady	Expert	LMV	NCI
	Imre Boros	Visiting Fellow	LMV	NCI
	Chou-zen Giam	Guest Researcher	LMV	NCI
	Kuan-Teh Jeang	Medical Staff Fellow	LMV	NCI
	Michael Nerenberg	Medical Staff Fellow	LMV	NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Molecular Virology

SECTION

Virus Tumor Biology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

3.4

PROFESSIONAL:

3.4

OTHER:

0

CHECK APPROPRIATE BOXES)

- (a) Human subjects (b) Human tissues (c) Neither
 (a1) Minors
 (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

This project is directed toward a molecular understanding of the enhancer elements and the trans-acting transcriptional factors that interact with enhancers. A recent focus of this project has been the role of the 3' long open reading frame of the human T-cell leukemia virus type-I (HTLV-I) which encodes a 40-Kd protein (p40x) that positively regulates transcription directed by the HTLV-I long terminal repeat (LTR) in a phenomenon known as trans-activation. We have succeeded in expressing the complete p40x coding sequence in *E. coli* and in baculovirus vector as a 40-Kd protein. Both p40x proteins are capable of stimulating transcription from HTLV-I LTR. Significant purification of the p40x proteins has been achieved. Our future objective is to understand the biochemical mechanism of trans-activation by the p40x protein and the involvement of cellular transcription factors in this process.

PROJECT DESCRIPTIONNames, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

George Khoury	Chief	LMV	NCI
John Brady	Expert	LMV	NCI
Imre Boros	Visiting Fellow	LMV	NCI
Chou-zen Giam	Visiting Fellow	LMV	NCI
Kuan-Teh Jeang	Medical Staff Fellow	LMV	NCI
Michael Nerenberg	Medical Staff Fellow	LMV	NCI

Objective:

This project concerns the mechanism of enhancer function in both B-lymphocytes and T-lymphocytes. A recent focus of this project has been on the molecular mechanism of transcriptional activation of the HTLV-I LTR as mediated by the HTLV-I p40^X protein. This study should yield information about the biochemical mechanism of p40^X action and the direct or indirect interactions between the enhancer element in the HTLV-I LTR and the p40^X protein. The current approaches include:

1. Purification of the p40^X protein from the expression systems presently available in the laboratories.
2. Characterization of the chemical structure of the p40^X protein by conventional techniques such as the sucrose gradient sedimentation, gel filtration chromatography, etc.
3. Introduction of the purified p40^X protein into tissue culture cells to assay for its function.
4. Use of both chemical and genetic methods to alter the structure of the p40^X protein to determine the relationship between its structure and its function.

Methods Employed:

Recombinant DNA techniques; bacterial expression vectors; somatic cell fusion techniques; protein purification; gene expression using transient and permanent assays; RNA and protein analysis.

Major Findings:

We have successfully expressed the p40^X protein in *E. coli* and eukaryotic cells in a baculovirus vector and showed that p40^X proteins are biologically active in transactivating transcription from the HTLV-I LTR. We have also devised simple fractionation procedures to purify the p40^X protein. Further studies will be developed in the area of the structure and function of the p40^X protein and the biochemical mechanisms of trans-activation.

Publications:

Giam, C. -Z., Nerenberg, M., Khoury, G. and Jay, G.: Expression of the complete HTLV-I p40^x coding sequence as a functional protein in Escherichia coli. Proc. Natl. Acad. Sci. USA (In Press)

Patents:

None

ANNUAL REPORT OF

THE LABORATORY OF TUMOR VIRUS BIOLOGY BIOLOGICAL CARCINOGENESIS PROGRAM DIVISION OF CANCER ETIOLOGY NATIONAL CANCER INSTITUTE

October 1, 1985 through September 30, 1986

The Laboratory of Tumor Virus Biology (1) identifies and characterizes exogenous viruses associated with the initiation or progression of neoplasia in humans or in animals as models for human neoplasia; (2) elucidates the mechanisms by which viruses associated with naturally-occurring cancers may induce or initiate neoplasia; (3) characterizes and defines the biology and molecular biology of viruses associated with naturally-occurring carcinomas; (4) identifies and characterizes factors involved in viral and cellular gene regulation pertinent to carcinogenesis; and (5) elucidates and defines the cellular and molecular basis of the transformation in carcinogenic progression.

The Viral Oncology Section (1) studies the molecular biology and gene regulation of the papillomaviruses and mechanisms of papillomavirus-induced transformation and carcinogenic progression; (2) develops techniques for DNA-mediated gene transfer; and (3) carries out studies on extrachromosomal plasmid replication, recombination, and partitioning in mammalian cells.

The Cellular Regulation and Transformation Section (1) examines the role of DNA tumor viruses in carcinogenesis and neoplasia; (2) characterizes the cellular and biochemical alterations associated with the oncogenic transformation; (3) analyzes the specific interactions between cellular and viral proteins during oncogenesis; and (4) examines the effect of specialized cellular differentiated functions on viral gene expression.

Among the primary objectives of the Laboratory of Tumor Virus Biology is the evaluation of the potential role of the human papillomaviruses in carcinogenesis. To this end we have investigated the molecular biology of the papillomaviruses. A major focus of this Laboratory has been the molecular biology of the bovine papillomavirus type 1 which is one of a subgroup of papillomaviruses which readily transform rodent cells in culture. This virus provides a useful model for the systematic study of the molecular biology and genetics of the papillomaviruses. The BPV-1 genome has been shown to encode two transforming genes, one mapping to the E6 open reading frame (ORF) and the second to the E5 ORF. In transformed mouse cells, the viral genome remains as a stable multicopy plasmid, which is apparently faithfully partitioned to daughter cells at cell division. BPV-1, therefore, has characteristics making it an excellent model for studying cellular transformation and latent viral infection functions.

We have mapped a transcriptional regulatory element within the 1 kilobase (kb) long control region (LCR) of the BPV-1 genome using an enhancer dependent expression vector for chloramphenicol acetyltransferase. This enhancer element works in a position- and orientation-independent manner and its function is transactivated

by the viral E2 gene product. We have examined the requirement for the E2 gene in viral transformation and replication assays, and have found that it is required for efficient cellular transformation and for stable plasmid maintenance.

We have shown that this function can be provided in trans. When the transformation functions are expressed from a surrogate promoter, however, the E2 gene product is no longer required indicating that its role in viral transformation is indirect. Its effect on transformation as well as on stable plasmid replication appears to be due to the transcriptional activation of an enhancer in the LCR which is required for the transcription of genes involved directly in transformation and replication.

A series of Bal 31 deletion mutants have been made through the viral LCR in order to define the precise target(s) of this transactivation. We have cloned these deletions in a forward and backward orientation into an enhancer-deleted expression vector and assayed the deletion mutants for their ability to respond to the E2 trans factor in an enhancer-dependent assay. We have defined two regions within the LCR which are activated by E2 expression. One maps in the vicinity of the 7185 promoter and manifests a fivefold activation in the presence of E2. The second region maps between bases 7611 and 7805. In the presence of E2 this region can activate transcription approximately 100-fold from a heterologous promoter. Preliminary results indicate that the promoters at base 89 and at base 7940 are responsive to this inducible enhancer element.

In the absence of a cell culture system for the productive replication of the papillomaviruses, detailed transcription analyses of these viruses have been hampered. A cDNA library has been constructed directly in an Okayama-Berg expression vector from RNA isolated from a BPV-1 fibropapilloma. Six distinct classes of cDNAs have been analyzed. Five of these six classes of cDNAs have 5' ends mapping near nucleotide 7250, indicating the presence of a major wart-specific promoter which is not active in transformed cells. Transcription in the fibropapilloma uses the same polyadenylation site used in transformed cells at approximately nucleotide 4200. In addition, there is a wart-specific polyadenylation site at nucleotide 7175 located downstream from the transforming region polyadenylation site. These additional wart-specific RNAs encode the L1 (major capsid) and L2 (minor capsid) proteins. One major class of late specific RNAs were found to encode the C terminus of E2 and another class could encode the E4 protein. Using sensitive S1 protection techniques as well as primer extension techniques with synthetic end-labeled oligonucleotides, the 5' termini of the mRNAs from bovine warts, as well as from BPV-1 transformed cells, have been analyzed. In addition to the wart-specific promoter mentioned above, additional promoters have been found to be active both in transformed cells as well as in warts. The 5' ends of the RNAs transcribed by these promoters in the LCR map to bases 7185, 7940, and 89. In addition, downstream from the LCR, 5' ends were found to map in the vicinity of bases 2443 and 3080. The presence of multiple promoter elements within the papillomavirus genomes indicate that transcription for the papillomaviruses is complex and probably highly controlled by viral as well as cellular factors.

Run on transcription *in vitro* done with isolated nuclei from transformed cells reveals that transcription proceeds through most of the late region downstream from the polyadenylation site utilized in the transformed cells. However, transcription appears to terminate near the 3' end of the late region upstream from the late polyadenylation site. It seems likely, therefore, that transcriptional termination may be a major cause of the "block" in late messenger RNA synthesis and, therefore, in virus production in transformed cells.

A line of transgenic mice harboring the complete BPV-1 genome has been developed by Doug Hanahan at the Cold Spring Harbor Laboratories in Cold Spring Harbor, NY. These mice develop dermal fibroblastic tumors at about 8 months of age. In addition to these tumors, the mice develop large areas of abnormal skin with hair loss due to a generalized proliferation of dermal fibroblasts, with atrophy of the overlying epidermis and loss of hair follicles. The BPV-1 genome is integrated in the normal tissues of these mice; however, multiple extrachromosomal copies of the DNA can be detected in the abnormal skin and in the dermal tumors of these mice. Examination of the tissues of these mice reveals that transcription can only be detected in the abnormal appearing skin and tumors of older mice. No transcription is detected in the internal organs of these mice. In addition, no viral RNA is found in the tissues of newborn or young mice with no skin abnormalities in which the DNA is still integrated. Although BPV-1 transcriptional activity and extrachromosomal replication appear to correlate well with dermal fibroblastic proliferation, these activities are not sufficient for tumor formation. We postulate that a second genetic event is necessary for tumor formation in these mice. Several cell lines have been established from the tumors and skin of these mice. The state of BPV-1 DNA as well as expression is identical in the cell lines as well as in the tissues from which the cell lines are established. Two cell lines established from one mouse differed in their growth properties. One cell line established from a tumor was not contact inhibited; the other from non-tumorous skin was flat and contact inhibited. Each of these cell lines, however, contains multiple extrachromosomal copies of BPV-1 and each expresses large amounts of BPV-1-specific RNA. Thus, the presence of BPV-1 transcriptional activity and the extrachromosomal plasmids are, by themselves, not sufficient for the fully transformed phenotype. A second genetic event is necessary for the fully transformed phenotype.

Mutagenesis studies have localized one of the two independent transforming genes of BPV-1 to the 3' half the E5 ORF. The E5 transforming protein of BPV-1 has been identified utilizing an antiserum generated against a synthetic peptide corresponding to the 20-carboxy terminal amino acids of the E5 ORF. The E5 polypeptide is the smallest viral or cellular transforming protein yet defined. It is 44 amino acids in size and has an apparent molecular weight of 7 kilodaltons (kd) on SDS polyacrylamide gels. The transforming polypeptide is predicted to be strikingly hydrophobic with 68% of the amino acids being hydrophobic. Cell fractionation studies have localized this polypeptide primarily to cellular membranes. The polypeptide exists as a dimer within cells and has a half-life of approximately 2 hours.

Studies on transcriptional transactivation and control elements have been extended to the human papillomaviruses. We have been particularly interested in HPV-16 which is one of the human papillomaviruses which has been directly associated with human cervical carcinoma. A conditional transcriptional enhancer has been identified in the LCR of the HPV-16 genome. This element functions in an orientation- and position-independent manner to activate the enhancer-deleted SV40 early promoter in the presence of the HPV-16 E2 gene product. Analyses have been done in acutely cotransfected monkey CV-1 cells in which the E2 gene of HPV-16 is expressed from the SV40 early promoter. Translational termination linkers cloned into the E2 ORF of the HPV-16 genome dramatically reduced the level of transcriptional transactivation. It has been shown that the analogous LCR enhancer element derived from BPV-1 can also be transactivated by the E2 gene product of HPV-16. Similarly, the HPV-16 LCR enhancer can be transactivated by the E2 gene product

from BPV-1. We have previously shown that two cervical carcinoma cell lines contain HPV-16 integrated into the host chromosome. The SiHa cell line contains a single copy of HPV-16 integrated, and the CaSki cell line contains 600 copies of the viral genome integrated. The single HPV-16 genome in the SiHa cell line was cloned in a 10 kb HindIII fragment which also contained flanking cellular DNA sequences. The site of integration was determined with respect to the virus and found that the integration disrupted the E2 and E4 ORFs. A series of host viral fusion ORFs were noted but transcriptional studies indicated that these ORFs were not transcriptionally active. The HPV-16 genome had integrated into an Alu-repeated sequence as determined by the sequence of the flanking host sequences. Analysis of the three most abundant BamHI clones from the CaSki cell line revealed that these consist of full length HPV-16 DNA, a 1.4 kb deletion of the LCR, and a 2.6 kb tandem repeat of the 3' transforming region or analogous region from the HPV-16 genome. Each of these abundant forms is repeated in a multiple head to tail tandem fashion within the genome. Northern blot analysis of the RNA from these two cervical carcinoma lines indicates that the HPV-16 genomes are transcriptionally active. In each case, utilizing subgenomic strand-specific probes, we have demonstrated that the transcripts are derived primarily from the E6 and E7 ORFs.

Another area of major investigation in the Laboratory has been the study of the mechanism of transformation by the murine polyoma virus. The transforming gene for this virus has been localized to the middle tumor antigen. It has been previously shown that this protein forms a complex with the cellular pp60c-src which is a tyrosine kinase. We have shown that the synthesis of pp60c-src in polyoma transformed cells can be regulated by the antisense c-src RNA expressed from a mouse metallothionein promoter. Clonal lines expressing complementary src RNA were found to grow at reduced rates in soft agar, form fewer foci and monolayers of normal rat cells, and form tumors more slowly after injection into syngeneic rats than parental cells. These studies demonstrate that the level of pp60c-src kinase activity affects the growth characteristics and transformation properties of polyomavirus-transformed cells. We have attempted to ask how general the phenomena of increased pp60c-src tyrosyl kinase activity is in transformed cells. Utilizing SV40, adenovirus 2, adenovirus 12, or BPV-1-transformed cells, we have shown that these transformed cells sporadically possess elevated pp60c-src kinase activity when compared to normal cells, and this increased kinase activity was a result of an apparent increase in specific activity of the protein rather than an increase in the level of the protein product itself. In these other transformed cells pp60c-src was not found physically associated with the tumor antigens known to be encoded by these viruses in contrast to polyomavirus transformed cells.

The role of pp60c-src in transformation has been extended from polyoma virus transformed cells to human tumors. We have observed a 20- to 40-fold increase in pp60c-src tyrosine protein kinase activity in human neuroblastoma cell lines over that observed in either human glioblastoma cell lines or human fibroblasts. The levels of c-src RNA or pp60c-src protein in these cells, however, are not significantly elevated over those levels found in glioblastoma cells. Additional human tumor tissues and human tumor cell lines have been analyzed for the level of pp60c-src protein kinase activity. All cell lines derived from tumors of neuroectodermal origin, which have a neural phenotype, express high levels of the c-src tyrosine-specific protein kinase activity. In contrast, cell lines derived from tumors of neuroectodermal origin that do not express neural characteristics, such as glioblastomas and melanomas, have pp60c-src molecules with low levels of protein kinase activity. These results in the cell lines are parallel to the

results seen in examining tumor tissues directly. Analysis of human tumor cell lines derived from tissues other than those of neuroectodermal origin are generally low with some notable exceptions. These exceptions include rhabdomyosarcoma, osteogenic sarcoma, Ewing's sarcoma, breast carcinoma, and colon carcinoma. Comparison of pp60c-src kinase activity in normal skeletal muscle and rhabdomyosarcoma tissue and in normal breast tissue and breast adenocarcinoma tissue revealed that pp60c-src activity was specifically elevated in the tumor tissues of both of these cases. These observations suggest that the phosphotransferase in some rhabdomyosarcomas and breast carcinomas may be a characteristic acquired during the malignant transformation of the cells.

The elevated activity of pp60c-src found in neuroblastomas, as compared to glioblastoma cells or normal human fibroblasts, is not due to the abundance of the pp60c-src protein or to specific and stable association with other cellular proteins. The pp60c-src molecules synthesized in neuroblastoma cells appear altered in their mobilities in SDS polyacrylamide gels when compared to the similar protein synthesized in glioblastoma cells. The biophysical basis for this different electrophoretic behavior has been localized to alterations within the amino terminal half of the protein. Cell-free translation of the cytoplasmic RNA from neuroblastoma cells and from glioblastoma cells, however, revealed that the *in vitro* synthesized proteins possess similar mobilities on SDS polyacrylamide gels and have indistinguishable protein kinase activities. There apparently is a specific post-translational mechanism in human glioblastoma cells which negatively regulates the pp60c-src kinase activity. This degree of negative regulation of the pp60c-src kinase activity in human neuroblastoma cells is diminished by neural differentiation.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE		PROJECT NUMBER
NOTICE OF INTRAMURAL RESEARCH PROJECT		Z01CP00543-08 LTVB
PERIOD COVERED October 1, 1985 to September 30, 1986		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Characterization of the Papillomaviruses		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
PI:	P. M. Howley	Chief LTVB NCI
Co-PI	C. Baker	Medical Officer LTVB NCI
Others:	B. Spalholz	Guest Researcher LTVB NCI
	V. Lindgren	Guest Researcher LTVB NCI
	P. Lambert	Guest Researcher LTVB NCI
	P. Hermonat	Guest Researcher LTVB NCI
	M. Sippola-Thiele	Visiting Fellow LTVB NCI
	J. Byrne	Biologist LTVB NCI
COOPERATING UNITS (if any) Dept. Physiological Chemistry, University of Wisconsin, Madison, WI (E. Schenborn, J. Dahlberg); Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (Doug Hanahan).		
LAB/BRANCH Laboratory of Tumor Virus Biology		
SECTION Viral Oncology Section		
INSTITUTE AND LOCATION NCI, NIH, Bethesda, MD 20892		
TOTAL MAN-YEARS:	5.6	PROFESSIONAL: 4.6 OTHER: 1.0
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p>The papillomaviruses are a group of small DNA viruses associated with benign and malignant proliferative lesions in a variety of higher vertebrates. Currently, there are recognized to be 41 distinct human papillomaviruses (HPVs) and six bovine papillomaviruses (BPVs). The lytic expression of these viruses is linked to the state of differentiation of squamous epithelial cells and to date no tissue culture system exists for their propagation in the laboratory. The bovine papillomavirus type 1 (BPV-1) is one of a subgroup of papillomaviruses which is capable of inducing fibroblastic tumors when innoculated into hamsters and is capable of inducing morphologic transformation of certain rodent cells in tissue culture. To date, transformation of rodent cells remains the only <i>in vitro</i> assay for the systematic study of the papillomaviruses. Because of this property, BPV-1 has become the prototype for unravelling the molecular biology of the papillomaviruses. A unique feature of this papillomavirus transformation system is that the viral DNA does not integrate into the host chromosome. The DNA remains extra-chromosomal as a stable multiple copy plasmid. The factors involved in stable transformation as well as for stable plasmid maintenance are being extensively studied. A second characteristic associated with the papillomavirus infection is the propensity of certain viruses to be associated with lesions which may progress to carcinomas. What factors, either viral or host, which are involved in such a progression from a benign lesion to a carcinoma are as yet unknown. Our studies are designed to unravel the molecular biology of the normal virus infection of cells as well as for understanding the viral and cellular factors involved in carcinogenic progression. We have determined that BPV-1 encodes at least two genes which can independently transform mouse cells. Also, we have mapped transcriptional regulatory elements in the LCR of BPV-1 which are <u>trans-activated</u> by the viral E2 gene product. We have been able to show that viral transformation and transcriptional transactivation map to distinct genes.</p>		

PROJECT DESCRIPTIONNames, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

P. M. Howley	Chief	LTVB, NCI
C. Baker	Medical Officer	LTVB NCI
B. Spa1holz	Guest Researcher	LTVB NCI
M. Sippola-Thiele	Visiting Fellow	LTVB NCI
V. Lindgren	Guest Researcher	LTVB NCI
P. Lambert	Guest Researcher	LTVB NCI
P. Hermonat	Guest Researcher	LTVB NCI
J. Byrne	Biologist	LTVB NCI

Objectives:

1. To analyze the molecular biology of the papillomaviruses using the bovine papillomavirus as a model system.
2. To localize and characterize the transforming regions of the bovine papillomavirus.
3. To localize and characterize the regions of the papillomavirus involved in latent plasmid replication.
4. To determine whether or not the transforming functions encoded by the bovine papillomavirus can complement other known viral or cellular oncogenes in transforming primary rodent cells.
5. To develop a tissue culture system for the propagation of papillomaviruses.
6. To analyze the viral mRNAs expressed in BPV-1-transformed cells as well as in productively infected fibropapillomas excised directly from cattle.
7. To localize the transcriptional promoters functional in productively and non-productively infected cells.
8. To determine the factors, both viral and cellular, involved in the control of "late" or virus-specific gene expression for the papillomaviruses.
9. To analyze the "early" papillomavirus protein products expressed in transformed cells.
10. To determine the cis and trans functions required for autonomous extrachromosomal plasmid replication.
11. To determine the nature of the molecular events involved in the progression of a benign papillomavirus lesion into a malignant lesion.
12. To characterize the virally encoded gene products involved in the transcriptional control of the papillomavirus genome.

Methods Employed:

1. Standard recombinant DNA technology for the cloning and propagation of papillomavirus hybrid plasmids.
2. Tissue culture.
3. Transcriptional analysis including Northern blotting, cDNA cloning into expression vectors, and nuclear run-off experiments.
4. DNA sequencing.
5. The generation of synthetic peptides based on DNA sequence information.
6. Immunoprecipitation.
7. Immunoblotting and immunofluorescence of viral proteins.

8. Transfer of DNA into mammalian cells using standard calcium precipitation, DEA dextran or electroporation technology.

Major Findings:

1. We have mapped a transcriptional regulatory sequence within the 1 kb LCR of the BPV-1 genome using an enhancer-dependent expression vector for chloramphenicol acetyltransferase (CAT). This transcriptional regulatory element works in a position- and orientation-independent manner, and its function is significantly augmented in BPV-1-transformed cells and in monkey CV-1 cells acutely cotransfected with plasmids expressing BPV-1 early gene products. Using defined deletion mutants of the BPV-1 genome and full-length viral cDNAs expressed from an SV40 early promoter, we demonstrate that the expression of this transactivating factor maps to the 3' ORFs of the viral transforming region. A premature termination codon engineered into the E2 ORF eliminates the expression of this diffusible transactivation function, establishing the E2 gene product as the diffusible transactivating factor. These studies have been published by Spalholz et al. (Cell 42: 183-191, 1985).
2. In collaboration with the Dahlberg laboratory at the University of Wisconsin, we have constructed a mutant of BPV-1 DNA that lacks a transcriptional enhancer located 3' to the polyadenylation site of the early viral RNAs expressed in transformed cells. This enhancer has previously been described by the Botchan laboratory at the University of California at Berkeley. This mutant DNA, lacking the distal enhancer, transformed mouse cells with an efficiency comparable to that of the full BPV-1 genome and it existed as a stable multiple copy plasmid in transformed cells. We conclude, therefore, that the BPV-1 distal enhancer is not cis-essential for the expression of viral genes involved in cellular transformation or plasmid maintenance. These data have been published (Howley et al., Mol. Cell. Biol. 5: 3310-3315, 1985).
3. BPV-1 encodes two independent transforming genes. One has previously been mapped to the E6 ORF and the second mapped to the 3' ORFs including E2, E3, E4, and E5. By targeting mutations to each of these specific ORFs by insertion of premature termination codon or use of base mutagenesis using synthesized oligonucleotides and M13 cloning, we have shown that the E5 gene is a transforming gene. In a wild type viral DNA background, the integrity of the E2 ORF is required for transformation but this effect is likely indirect due to the requirement of E2 for presumed transcriptional activation of the conditional enhancer elements located in the LCR. We have shown that a cDNA containing the 3' ORFs intact and expressed from the SV40 early promoter can induce transformation of C127 cells and can transactivate the LCR inducible enhancer. Mutagenesis of this cDNA behind the surrogate SV40 promoter has permitted the dissociation of the transformation and transactivation functions to the E5 and E2 ORFs, respectively (Yang et al., Nature 318: 575-577, 1985). A paper describing the requirement for an intact E2 gene for efficient transformation and plasmid replication is now in press (Rabson et al., J. Virol.).
4. A line of transgenic mice harboring the complete BPV-1 genome has been developed by Doug Hanahan at the Cold Spring Harbor Laboratories. These mice develop dermal fibroblastic tumors at about 8 months of age. In addition to these

tumors, the mice develop large areas of abnormal skin with hair loss due to a generalized proliferation of dermal fibroblasts, with atrophy of the overlying epidermis and loss of hair follicles. The BPV-1 DNA is integrated into the normal tissues of these mice in that multiple extrachromosomal copies of the DNA can be detected in the abnormal skin and in the dermal tumors.

We have examined the tissues from these transgenic mice to analyze expression of the BPV-1 genome. BPV-1 transcripts are readily detected in the abnormal appearing skin and tumors of older mice, but not in the internal organs of these same mice. Also, no viral RNA was found in tissues of newborn or young mice with no skin abnormalities. Thus, although BPV-1 transcriptional activity and extrachromosomal plasmid replication correlate well with dermal fibroblastic proliferation, these activities are not sufficient for tumor formation. We, therefore, postulate that a second genetic event is necessary for tumor formation.

Several cell lines have been established from the tumors and skin of the mice. The state of the BPV-1 DNA, as well as its expression, was identical in the cell lines and in the tissues from which they were established. Two cell lines established from one mouse differed in their growth properties. One cell line established from a tumor was not contact inhibited; the other from nontumorous skin was flat and contact inhibited. Each of these cell lines contains multiple extrachromosomal copies of BPV-1 DNA and each expresses BPV-1-specific RNA. Thus, the presence of BPV-1 transcriptional activity and extrachromosomal plasmids are not sufficient for the fully transformed phenotype. We are currently attempting to identify what factors may contribute to neoplastic progression and to determine the genetic and molecular basis of this progression.

5. We have previously shown that the 1.0 kb LCR of the bovine papillomavirus (BPV-1) genome contains a transcriptional enhancer which is markedly augmented in the presence of the viral E2 gene product(s) (Spalholz et al., Cell 42: 183-191, 1985). To define the precise target(s) of this transactivation, we have generated a series of deletions using Bal 31 digestion between the HindIII site at base 6958 and the HpaI site at base 7945 of the BPV-1 LCR. We have cloned these deletions in the forward and backward orientations into the unique BglII site of pA₁₀CAT. Results from cotransfections with an E2 expressing plasmid in monkey CV-1 cells indicate that two enhancer regions exist in the LCR which are activated by E2 expression. One region maps in the vicinity of the 7185 promoter (P7185) and manifests a fivefold activation in the presence of E2 in a CAT assay. A second region maps between nt 7611 and 7805. In the presence of E2 this region can activate transcription approximately 100-fold from a heterologous promoter. We have also made a series of BPV-1 LCR promoter CAT constructs to ascertain which BPV-1 promoters may be responsive to the E2 mediated enhancer activation. Preliminary results indicate that the promoters P89 and P7940 are both responsive. A paper describing these data is in preparation.

Publications:

Howley, P. M.: Human papillomaviruses: Present day knowledge and future approaches. Clinics in Dermatology 3: 24-211, 1985.

Howley, P. M., Schenborn, E. T., Lund, E., Byrne, J. C. and Dahlberg, J. E.: The bovine papillomavirus distal "enhancer" is not cis-essential for transformation or for plasmid maintenance. Mol. Cell. Biol. 5: 3310-3315, 1985.

Howley, P. M., Yang, Y.-C., Spalholz, B. and Rabson, M. S.: Molecular aspects of papillomavirus-host cell interactions. Banbury Report 21: Viral Etiology of Cervical Cancer. New York, Cold Spring Harbor Laboratory, 1986, pp. 261-272.

Howley, P. M., Yang, Y.-C., Spalholz, B. A., and Rabson, M. S.: Papillomavirus transforming functions. CIBA Foundation Symposium No. 120: Papillomaviruses. p. 39-47, 1986.

Rabson, M. S., Yang, Y.-C., and Howley, P. M.: A genetic analysis of bovine papillomavirus type 1 transformation and plasmid maintenance functions. Cancer Cells. New York, Cold Spring Harbor Laboratory. (In Press)

Rabson, M.S., Yee, C., Yang, Y.-C., and Howley, P. M.: Analysis of the bovine papillomavirus type 1 3' early region transformation and plasmid maintenance functions. J. Virol. (In Press)

Spalholz, B. A., Yang, Y.-C., Howley, P. M.: Identification of a new enhancer within the non-coding region of BPV-1 which is trans-activated by BPV-1 early gene products. In Howley, P. M. and Broker, T. R. (Eds.): UCLA Symposia on Molecular and Cellular Biology, Papillomaviruses: Molecular and Clinical Aspects. New York, Alan R. Liss, Inc., 1985, Volume 32, pp. 343-362.

Spalholz, B. A., Yang, Y.-C. and Howley, P. M.: Transactivation of a bovine papillomavirus transcriptional regulatory element by the E2 gene product. Cell 42: 182-191, 1985.

Yang, Y.-C., Okayama, H., and Howley, P. M. Structural analysis of viral cDNAs from bovine papillomavirus transformed mouse cells. In Howley, P. M. and Broker, T. R. (Eds.): UCLA Symposia on Molecular and Cellular Biology, Papillomaviruses: Molecular and Clinical Aspects. New York, Alan R. Liss, Inc., 1985, Volume 32, pp. 363-368.

Yang, Y.-C., Spalholz, B. A., Rabson, M. S., and Howley, P. M.: Dissociation of transforming and trans-activation functions for bovine papillomavirus type 1. Nature 318: 575-577, 1985.

Patents:

None

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01CP00547-06 LTVB

PERIOD COVERED

October 1, 1985 to September 30, 1986

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

The Use of Papillomavirus DNAs as Eukaryotic Cloning Vectors

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	P. M. Howley	Chief	LTVB	NCI
Others:	D. Levens	Medical Staff Fellow	LP	NCI
	P. Hermonat	Guest Researcher	LTVB	NCI

COOPERATING UNITS (if any)

Revlon Health Care Research and Development, Springfield, VA (N. Sarver).

LAB/BRANCH

Laboratory of Tumor Virus Biology

SECTION

Viral Oncology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:

0.8

PROFESSIONAL:

0.8

OTHER:

0.0

CHECK APPROPRIATE BOX(ES)

- (a) Human subjects (b) Human tissues (c) Neither
- (a1) Minors
- (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The bovine papillomavirus (BPV) is capable of transforming certain rodent fibroblast lines in which the viral DNA remains as a stable extrachromosomal plasmid. These properties have been exploited in developing BPV into a stable extrachromosomal mammalian cell vector. The complete genome cloned into pML2, which is a deletion derivative of pBR322, is capable of serving as a shuttle vector which can replicate as a plasmid in mouse C127 cells or in bacteria. We have studied the expression of the rat preproinsulin gene in BPV vectors in C127 cells and have shown that the expression of the gene is enhancer dependent. An "enhancer" element is located in the BPV-1 genome at the 3' end of the transforming region, downstream from the polyadenylation recognition sequence. Using this vector system, a variety of exogenous genes have been expressed. A portion of the human T-cell lymphotropic virus type 1 (HTLV-1) has been expressed off of the mouse metallothionein promoter in a BPV vector. The extrachromosomal state of the DNA should provide a physical characteristic to permit the purification of chromatin complexes of mammalian genes. Using the lac operator, we have developed a technique for rapid purification and identification of sequence-specific binding proteins. This technique, combined with the extrachromosomal papillomavirus plasmid vector system, should facilitate the identification of important viral and cellular gene regulatory proteins.

PROJECT DESCRIPTIONNames, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

P. M. Howley	Chief	LTVB	NCI
D. Levens	Medical Staff Fellow	LP	NCI
P. Hermonat	Guest Researcher	LTVB	NCI

Objectives:

This project is directed toward the development of the papillomaviruses as effective and useful mammalian cell vectors. In studying the expression of a variety of foreign genes in papillomavirus based vectors, information is also generated concerning the molecular biology and genetic organization of papillomaviruses.

Methods Employed:

Recombinant-DNA techniques, nucleic acid hybridization, gel electrophoresis, tissue culture, RNA analysis, DNA transfection techniques, radioimmunoassays, immunoprecipitation.

Major Findings:

1. The effect of position in a BPV-1 vector on foreign gene expression was assessed using the rat preproinsulin gene as a model. This gene was inserted at each of the two BPV-1/pML2 junctions in either transcriptional orientation in the pdBPV-1(142-6) shuttle vector previously described by this laboratory. Mouse cells containing this plasmid shuttle vector were selected solely on the basis of morphological transformation and then assayed for rat preproinsulin gene expression. Cells containing this gene at the 3' end of the BPV-1 transforming region expressed the rat preproinsulin gene, whereas cells that contain the gene at the 5' end of the nontransforming region did not. Variability in the plasmid copy number or the extent of DNA rearrangements could not account for this difference. We conclude that the expression of the rat preproinsulin gene in mouse cells using BPV-1 vectors depends on the transcriptional activation afforded by viral (enhancer) sequences located at the 3' end of the transforming region. An enhancer element has previously been localized to this region. Intervening BPV-1 or pML2d sequences appear to block this enhancer-mediated gene activation. In agreement with this viral enhancer dependent activation, a rat preproinsulin gene in the "blocked" position at the 5' end of the nontransforming region could be activated by the insertion of a DNA fragment containing the SV40, MSV, or BPV-1 enhancer element adjacent to the rat preproinsulin gene. Thus, a gene which is normally not expressed in a particular cell may be activated when placed adjacent to a viral enhancer in a BPV-1 vector. This work has been published (Sarver et al., Mol. Cell. Biol. 5: 3507-3516, 1985)

2. A bovine papillomavirus vector has been used to express a portion of a proviral DNA of a human T-cell leukemia (lymphotropic) virus (HTLV-1). The 3' end of the proviral DNA was inserted into a BPV-1 vector and expressed off a mouse

metallothionein promoter. Mouse cells were transfected with this vector and selected solely by virtue of morphologic transformation. These cells were analyzed by Northern blot analysis and indirect immunofluorescence and shown to express HTLV-1 viral gene products. These mouse cells were injected into Balb/C mice and a monoclonal antibody was recovered which specifically recognizes a 21 Kd protein present in HTLV-1 virions, indicating that the 3' end of the proviral DNA encodes the small envelope protein for the virus. A paper describing these results, done in collaboration with Marvin Reitz of the Laboratory of Tumor Cell Biology has been published (Eiden et al., Mol. Cell. Biol. 5: 3320-3324, 1985).

3. We have developed a general method for the enrichment and identification of sequence-specific DNA binding protein. This method utilizes the cloning of the DNA sequence of interest adjacent to the lac operator and using a lac repressor-beta-galactosidase fusion protein to fish out this DNA and any associated proteins from crude cellular extracts or fractions thereof. The utility of this technique has been demonstrated in bacteria to purify the lambda repressor and in purified preparations of the yeast mitochondrial RNA polymerase to identify a 70,000 MW peptide which binds specifically to the promoter region of the yeast mitochondrial 14S rRNA gene. This approach should have general utility in fishing out extra-chromosomal plasmids and their associated proteins and should have direct utility with BPV-1 based plasmids. A paper describing these results has been published (Levens and Howley, Mol. Cell. Biol. 5: 2307-2315, 1985).

Publications:

Eiden, M., Newman, M., Fisher, A., Mann, D., Howley, P., and Reitz, M.: HTLV-1 small envelope protein is expressed in mouse cells using a bovine papilloma virus-derived shuttle vector. Mol. Cell. Biol. 5: 3320-3324, 1985.

Howley, P. M.: Bovine papillomavirus vectors. In Fields, B. and Martin, M. (Eds): Genetically Altered Viruses and the Environment. New York, Cold Spring Harbor Laboratory, 1985, pp. 301-312.

Levens, D. L. and Howley, P. M.: A novel method for identifying sequence specific DNA binding proteins. Mol. Cell. Biol. 5: 2307-2315, 1985.

Sarver, N. and Howley, P. M.: Bovine papillomavirus DNA vectors. In Notkins, A. L. and Oldstone, M. B. A (Eds.): Concepts in Viral Pathogenesis. New York, Springer-Verlag. (In Press)

Sarver, N., Muschel, R., Byrne, J. C., Khoury, G., and Howley, P. M.: Enhancer dependent expression of the rat preproinsulin gene in bovine papillomavirus type 1 vector. Mol. Cell. Biol. 5: 3507-3516, 1985

Patents:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01CP00564-04 LTVB
PERIOD COVERED October 1, 1985 to September 30, 1986		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Early Events in Virus/Host Cell Interaction		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
PI:	R. Schlegel	Chief, Cellular Reg. and Trans. Section LTVB NCI
Others:	M. Glass	Biologist LTVB NCI
COOPERATING UNITS (if any) None		
LAB/BRANCH Laboratory of Tumor Virus Biology		
SECTION Cellular Regulation and Transformation Section		
INSTITUTE AND LOCATION NCI, NIH, Bethesda, MD 20892		
TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
.2	0.1	0.1
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p>Many animal viruses enter their respective host cells by the process of adsorptive endocytosis. This route of internalization is also used by many hormones and ligands which bind to specific receptors on the cell plasma membrane. After binding to the cell surface, viruses can follow different pathways dependent upon their particular structure and organization. For example, vesicular stomatitis virus (an enveloped, RNA virus) enters an acidic endosomal vesicle and, consequent to exposure to the acidic pH, is believed to fuse with the membrane of the vesicle. Fusion results in the release of viral nucleic acid into the cell cytoplasm. In contrast to VSV, SV40 (a nonenveloped, DNA virus) appears to translocate quickly from the endosomal vesicle to the nucleus where it is uncoated and its DNA released. The mechanism of this specific and rapid translocation is not known. Our laboratory is studying the mechanism by which two viruses (VSV and SV40) are "targeted" to different and specific sites in the host cell. Specifically, we have asked: (1) are these specific binding sites for viruses at the cell surface; (2) what are the cellular and viral components which participate in binding; (3) do the binding sites contribute to the final "destination" of the virus; (4) how do viruses cross cell membrane barriers; and (5) do viruses contain "signals" to direct their intracellular translocation? Multiple approaches will be used in these studies. Radio-labelled virus will be used to permit the detection and characterization of specific viral binding as well as the fate of internalized virus. Cell fractionation, electron microscopy, and immunofluorescence microscopy will also be used to follow the pathway of viral infection. Fusion of virus with cell membranes will be monitored by fluorescence energy transfer and EM of in vitro fusion models. Attempts will be made to delineate specific, biologically-active domains of viral spike and capsid proteins by use of both synthetic peptides and their corresponding antibodies. This project has been discontinued as of January 1986.</p>		

PROJECT DESCRIPTIONNames, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

R. Schlegel	Chief, Cellular Reg. and Trans. Section	LTVB	NCI
M. Glass	Biologist	LTVB	NCI

Objectives:

1. Define the number and composition of cell binding sites for VSV and SV40.
2. Identify domains of VSV spike protein and SV40 capsid proteins involved in:
 - a) cell binding
 - b) membrane fusion or disruption
 - c) targeting to the nucleus
3. Determine whether both VSV and SV40 can fuse or interact with cell membranes.

Methods Employed:

1. S^{35} -VSV binding assays.
2. Electron microscopy (EM).
3. Immunofluorescence microscopy (IF).
4. Liposome and cell fusion assays monitored by energy transfer fluorescence and fluorescence quenching.
5. Detection of G-protein conformation changes by circular dichroism and infrared spectroscopy.
6. Density gradient centrifugation.
7. Hemolysis assays.

Major Findings:

1. The spike protein of VSV can be reconstituted functionally into liposomes.
2. The spike protein of VSV mediates membrane fusion.
3. VSV spike protein interacts specifically with acidic phospholipids.
4. The NH_2 terminus of VSV spike protein is a pH-dependent hemolysin, hemagglutinin, and cytotoxin.
5. The terminal 6 amino acid peptide of VSV spike protein is the smallest known peptide hemolysin.
6. The terminal 6 amino acid peptide of VSV spike protein causes membrane destabilization and alters membrane permeability to ions.

Publications:

Schlegel, R.: Membrane-active peptides of the vesicular stomatitis virus glycoprotein. In Lonberg-Holm, K. and Crowell (Eds.): Virus Attachment and Entry into Cells. Washington, D. C., ASM Publications, 1986, pp. 66-73.

Schlegel, R.: Probing the function of viral fusion proteins with synthetic peptides. In Sowers, A. (Ed.): Cell Fusion. New York, Plenum Press. (In Press)

Patents: None

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01CP00565-04 LTVB

PERIOD COVERED

October 1, 1985 to September 30, 1986

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Transforming Activities and Proteins of the Papillomaviruses

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	R. Schlegel	Chief, Cellular Reg. and Trans. Section	LTVB	NCI
Others:	J. Bubb	Visiting Fellow	LP	NCI
	Y. Zhang	Visiting Fellow	LTVB	NCI
	A. Burkhardt	Staff Fellow	LTVB	NCI
	M. Glass	Biologist	LTVB	NCI

COOPERATING UNITS (if any)

Department of Human Genetics, Yale University, School of Medicine;
New Haven, Connecticut (Dr. Daniel DiMaio)

LAB/BRANCH

Laboratory of Tumor Virus Biology

SECTION

Cellular Regulation and Transformation Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:

4.8

PROFESSIONAL:

3.9

OTHER:

0.9

CHECK APPROPRIATE BOX(ES)

- (a) Human subjects (b) Human tissues (c) Neither
- (a1) Minors
- (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Papillomaviruses induce benign tumors in a variety of vertebrate species including man. These benign tumors, papillomas, can arise on a variety of squamous epithelial surfaces such as skin, larynx, and genitalia. In some cases, these benign viral-induced lesions can progress to carcinomas. Papillomas induced by the Shope papillomavirus (in rabbits) or the bovine papillomavirus type 4 (in cattle) can develop into carcinomas, and in humans the occurrence of cervical dysplasia and its progression to cervical carcinoma is closely associated with human papillomavirus types 16 and 18. In both animals and man it is believed that the papillomaviruses interact with additional factors (e.g., carcinogens and/or tumor promoters) to effect the complete transformed phenotype. The intent of our laboratory's investigations is to define the mechanism by which papillomaviruses transform immortalized cells in vitro and to determine how they contribute to tumorigenesis in vivo. Specifically, we are studying the transforming activities of cloned papillomavirus DNA as determined by focus assays and tumorigenicity in nude mice. We are also committed to identifying and characterizing the transforming proteins encoded by these viruses. To date, we have been able to demonstrate that the bovine papillomavirus effects the synthesis of an unusually small transforming polypeptide. This small protein, which is the product of the E5 open reading frame, is hydrophobic and is localized in cellular membranes. We have also been able to demonstrate that human papillomavirus DNA can convert NIH 3T3 cells to the tumorigenic state.

PROJECT DESCRIPTIONNames, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

R. Schlegel	Chief, Cellular Reg. and Trans. Section	LTVB	NCI
J. Bubb	Visiting Fellow	LP	NCI
Y. Zhang	Visiting Fellow	LTVB	NCI
A. Burkhardt	Staff Fellow	LTVB	NCI
M. Glass	Biologist	LTVB	NCI

Objectives:

1. Generate specific antisera against the early proteins of several papillomaviruses, including the bovine papillomavirus.
2. Isolate and characterize the biochemical and biological properties of the E5 and E6 early transforming proteins.
3. Perform mutagenesis studies of isolated transforming genes to determine the functional domains of the viral transforming proteins.
4. Use type-specific viral antisera to screen and evaluate the presence of human papillomaviruses in surgical pathology biopsies and specimens.
5. Establish an *in vivo* assay to evaluate the biological consequences of papillomavirus infection and the ability of papillomaviruses to collaborate with carcinogens and/or promoters to effect tumor progression.
6. Determine whether or not papillomaviruses activate or cooperate with cellular oncogenes to initiate tumor formation and progression.
7. Determine whether or not any of the early papillomavirus proteins function as TSTA molecules or whether they might be useful for immunoprophylaxis.
8. Determine whether or not human cervical carcinoma cell lines contain dominant transforming genes, and, if they do, are they identical to, or associated with, the integrated HPV DNA.

Methods Employed:

1. Keratinocyte tissue culture.
2. Electroporation of epithelial cells.
3. Recombinant DNA methodology for the construction of BPV and HPV molecules.
4. Immunoprecipitation, immunoblotting, and immunofluorescence of viral proteins.
5. DNA and RNA hybridization.
6. FACS analysis of cellular DNA content and cell surface tumor antigens.
7. Polyacrylamide and agarose gel electrophoresis.
8. DNA sequencing.
9. Site-specific mutagenesis using M13 vectors.
10. HPLC and FPLC of viral and cellular proteins.
11. Two dimensional gel electrophoresis.
12. Microinjection of tissue culture cells.

Major Findings:

1. We have identified the E5 transforming protein of bovine papillomavirus type 1 by means of an antiserum against a synthetic peptide corresponding to the 20

carboxy-terminal amino acids of the E5 ORF. The E5 polypeptide is the smallest viral transforming protein yet characterized; it has an apparent molecular weight

of 7 kd on SDS-polyacrylamide gels and a predicted molecular weight of 6 kd (44 amino acids). The transforming polypeptide is encoded entirely within the second half of the E5 open reading frame and its predicted amino acid composition is very unusual; 68% of the amino acids are strongly hydrophobic and 34% are leucine. Cell fractionation studies localized this polypeptide predominantly to cellular membranes. This polypeptide exists as a dimer within cells and has a half-life of approximately 2 hours.

2. We have shown that HPV DNA can convert NIH 3T3 cells to the tumorigenic state. Interestingly, this conversion is not necessarily accompanied by gross morphological transformation of the fibroblasts.

3. Genomic DNA from human cervical cell lines is capable of converting NIH 3T3 cells to the tumorigenic state and this conversion is accompanied by the transference of HPV DNA (in primary transfectants).

Publications:

Howley, P. M. and Schlegel, R.: Papillomavirus transformation. In Howley, P. M. and Salzman, N. (Eds.): The Papillomaviruses. New York, Plenum Press. (In Press)

Schlegel, R., Glass, M., Rabson, M. S., and Yang, Y.-C.: The E5 transforming gene of bovine papillomavirus directs synthesis of a small, hydrophobic polypeptide. Science. (In Press)

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01CP00898-03 LTVB

PERIOD COVERED

October 1, 1985 to September 30, 1986

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Role of Human Papillomaviruses in Human Carcinogenesis

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	P. M. Howley	Chief	LTVB	NCI
Co-PI:	C. C. Baker	Medical Officer	LTVB	NCI
Others:	R. Schlegel	Chief, Cellular Reg. and Trans. Section	LTVB	NCI
	V. Lindgren	Guest Researcher	LTVB	NCI
	W. Phelps	Guest Researcher	LTVB	NCI
	C. Yee	Biologist	LTVB	NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Tumor Virus Biology

SECTION

Viral Oncology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:

3.0

PROFESSIONAL:

2.0

OTHER:

1.0

CHECK APPROPRIATE BOX(ES)

- (a) Human subjects (b) Human tissues (c) Neither
- (a1) Minors
- (a2) Interviews

SUMMARY OF WORK (Use standard unraduced type. Do not exceed the space provided.)

The papillomaviruses are associated with naturally occurring carcinomas in a variety of species, including man. There are now 41 human papillomaviruses (HPVs) which have been identified in man. Six of these, HPV-6, HPV-11, HPV-16, HPV-18, HPV-31 and HPV-33, have been associated with human genital tract lesions. HPV-6, HPV-11, and HPV-31 have been found to be associated with a high percentage of benign genital warts, including cervical flat warts and dysplasias. HPV-16, HPV-18, and HPV-31 were each cloned directly from cervical carcinoma biopsy specimens and have been found in a high percentage of cervical carcinomas. We have previously screened a series of human carcinoma cell lines for the presence of human papillomavirus DNA sequences using HPV-6, HPV-11, HPV-16, and HPV-18 DNA probes. Six of eight cell lines which had been derived from human cervical carcinoma lines were found to contain integrated HPV DNA sequences. In five of these lines, HPV-specific polyadenylated RNA species could be identified. Two of the cell lines contained integrated HPV-16 DNA and in each of these cell lines the genomes were transcriptionally active. Genomic clones have been made from these HPV-16 positive lines and have been characterized. In the SiHa cell line in which only a single copy of the HPV-16 genome is integrated, the cellular flanking sequences have been sequenced. Integration has occurred in the E2 ORF of the HPV-16 genome. We have characterized a conditional enhancer in the control region of the HPV-16 genome and have shown that the E2 gene of HPV-16 encodes a transcriptional transactivating function that induces this enhancer element.

PROJECT DESCRIPTIONNames, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

P. M. Howley	Chief	LTVB	NCI
C. C. Baker	Medical Officer	LTVB	NCI
R. Schlegel	Chief, Cellular Reg. and Trans. Section	LTVB	NCI
V. Lindgren	Guest Researcher	LTVB	NCI
W. Phelps	Guest Researcher	LTVB	NCI
C. Yee	Biologist	LTVB	NCI

Objectives:

1. To analyze human squamous cell carcinomas from a variety of sites for the presence of HPV DNAs.
2. To analyze human squamous cell carcinomas for the presence of HPV-specific mRNAs.
3. To clone the integrated HPV DNA sequences from positive carcinomas in order to determine the site of integration with respect to the virus DNA as well as with respect to the host cellular DNA.
4. To identify the chromosomes into which the HPV DNAs have integrated.
5. To generate full-length cDNA clones of the viral transcripts from the positive carcinoma lines.
6. To determine which viral genes are being expressed within the cervical carcinomas and cervical carcinoma cell lines.
7. To determine whether hybrid viral-host cell RNAs are expressed within the positive carcinoma lines.
8. To determine whether HPV-16 and HPV-18 contain genes which can transactivate transcriptional regulatory sequences within the viral genome and within the host cell.

Methods Employed:

1. Standard recombinant DNA technology.
2. Northern blot analysis of RNAs.
3. cDNA cloning using expression vectors.
4. Immunoblotting and immunofluorescence of viral proteins.
5. DNA sequencing.
6. In situ hybridization.
6. Transient and stable DNA transfection techniques.

Major Findings:

1. We have cloned and analyzed the integrated HPV-16 genomes present in the human cervical carcinoma cell lines SiHa and CaSki. The single HPV-16 genome in the SiHa line has been cloned as a 10 kb HindIII fragment. Integration occurs at n. 3132 and 3384 of the HPV-16 genome with disruption of the E2 and E4 ORFs. An additional 52 bp deletion of HPV-16 sequences fuses the E2 and E4 ORFs. The 5' portion of the disrupted E2 ORF terminates immediately after entering the human right flanking region. Two human ORFs within the left human flank are fused with the E2 and E4 ORFs to produce human/E2 ORF and human/E4/E2 fusion ORFs coding for

123 and 152 amino acids, respectively. No other changes in the HPV-16 genome are detectable by heteroduplex analysis with the prototype HPV-16 DNA. Sequencing of the E1 ORF, however, has revealed the presence of an additional G at nt 1138 which fuses the E1a and E1b ORFs into a single ORF. Sequence analysis of the human flanking sequences has revealed a half Alu sequence at the left junction and a sequence highly homologous to the human O-repeat in the right flanking region.

Analysis of the three most abundant BamHI clones from the CaSki line showed that these consist of full length HPV-16 DNA, a 1.4 kb deletion of the LCR and a 2.6 kb tandem repeat of the 3' transforming region. These HPV-16 genomes are arranged in head to tail tandem repeats.

Northern blot analysis has revealed that the HPV-16 genome continues to be expressed in both cervical carcinoma cell lines, albeit at significantly different levels. Preliminary mapping with subgenomic strand-specific probes indicates that viral transcription appears to be primarily derived from the E6 and E7 ORFs. Currently, cDNA libraries are being made to study the RNA transcripts in more detail. A manuscript describing these integrated HPV-16 DNA sequences and their transcription is in preparation.

2. A conditional transcriptional enhancer has been identified in the long control region (LCR) of the HPV-16 genome. The HPV-16 LCR segment (nt7007 to nt57) functions in an orientation- and position-independent manner to activate the enhancer-deleted SV40 early promoter in pA₁₀CAT in the presence of an HPV-16 early gene product. Analysis of RNA from acutely transfected monkey CV1 cells confirms that this enhancement is due to transcriptional activation of the SV40 early promoter. The transactivation function has been mapped to the E2 open reading frame by the insertion of translational termination linkers into the early open reading frames of the HPV-16 genome.

Furthermore, it has also been shown that the analogous LCR enhancer derived from BPV-1 can be transactivated by the E2 gene product derived from HPV-16. Similarly, the HPV-16 LCR enhancer may be transactivated by the E2 gene product from BPV-1. Finally, several human carcinoma cell lines, previously demonstrated to harbor and express HPV sequences, have been assessed for the presence of an endogenous transactivation factor. A manuscript describing this work is in preparation.

Publications:

None

Patents:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE		PROJECT NUMBER
NOTICE OF INTRAMURAL RESEARCH PROJECT		201CP05420-02 LTVB
PERIOD COVERED October 1, 1985 to September 30, 1986		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Transformation by Polyomaviruses		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
PI:	J. B. Bolen	Senior Staff Fellow LTVB NCI
Others:	S. Amiri	Visiting Fellow LTVB NCI
	V. DeSeau	Biologist LTVB NCI
COOPERATING UNITS (if any) Dept. of Microbiology, State University of New York at Stony Brook, Stony Brook, NY (J. S. Brugge); Dept. of Molecular and Cellular Biology, Pennsylvania State University, University Park, PA (D. Shalloway)		
LAB/BRANCH Laboratory of Tumor Virus Biology		
SECTION Cellular Regulation and Transformation Section		
INSTITUTE AND LOCATION NCI, NIH, Bethesda, Maryland 20892		
TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.5	1.0	0.5
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p>The polyomaviruses comprise a class of small DNA tumor viruses within the papovavirus group of DNA viruses. Members of the polyomavirus class include polyoma virus (Py) of mice, simian virus 40 (SV40) of monkeys, and JC and BK viruses of humans. Of these viruses, Py has been most thoroughly characterized with respect to the genetic elements and proteins involved in oncogenic transformation of mammalian cells. Genetic and biochemical analysis has revealed that the region of the Py genome required for both Py-mediated tumorigenesis in vivo and oncogenic transformation in vitro encodes three nonstructural proteins: the large, middle, and small tumor antigens (T Ags). These proteins have been designated as T Ags because they are recognized by antibodies in sera from animals bearing Py-induced tumors (T sera). Of these three proteins, a central role for the Py-encoded middle T Ag (MTAg) in Py-mediated oncogenesis has been established principally through genetic analysis. The MTAg is associated with a protein kinase activity which can be detected by the in vitro phosphorylation of tyrosine on MTAg when T sera immunoprecipitates of Py-infected or Py-transformed cell extracts are incubated with gamma-32P ATP and Mg⁺⁺. The MTAg apparently does not possess intrinsic protein kinase activity and is thought to associate with the cellular c-src gene product, the cellular homolog of the Rous sarcoma virus transforming gene. Since both the viral (pp60v-src) and cellular (pp60c-src) forms of the src gene protein possess intrinsic tyrosine protein kinase activity, it has been proposed that the MTAg associated protein kinase represents a property of pp60c-src. The potential importance of this protein kinase activity in Py-mediated oncogenesis is suggested by the finding that viral mutants which are deficient in transforming potential generally lack this activity and that there are no known transformation-competent Py strains which encode MTAg molecules which do not possess this associated kinase activity.</p>		

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

J. B. Bolen	Senior Staff Fellow	LTVB	NCI
S. Amini	Visiting Fellow	LTVB	NCI
V. DeSeau	Biologist	LTVB	NCI

Objectives:

1. Role of polyomavirus middle tumor antigen and pp60c-src in polyomavirus transformation.
2. Role of pp60c-src in transformation of cells by other DNA tumor viruses.

Methods Employed:

1. Standard recombinant DNA technology for the cloning and propagation of Py chimeric plasmids.
2. Cell culture.
3. Cloning of mammalian cells.
4. Transfer of DNA into mammalian cells.
5. Transcriptional analyses of RNA including Northern and S1 analysis.
6. Immunoprecipitation analysis and protein kinase assays.
7. One and two-dimensional peptide analysis.
8. Two-dimensional phosphoamino acid analysis.
9. Immunoblot analysis.
10. High and medium pressure liquid chromatography of proteins and amino acids.
11. One and two-dimensional gel electrophoresis.
12. In vitro transcription and translation of cellular and viral mRNAs.
13. Generation of peptide-specific and amino acid-specific antisera.

Major Findings:

The synthesis of pp60c-src in Py-transformed cells can be regulated by antisense c-src RNA. In order to determine the potential role of elevated levels of pp60c-src kinase activity in polyomavirus transformed cells, we constructed a recombinant plasmid with the mouse metallothionein -1 promoter upstream of a src gene in an antisense orientation and cotransfected this plasmid into FR 18-1 cells (FR3T3 cells transformed by a plasmid capable of encoding only the polyomavirus middle tumor antigen) with a plasmid containing the neomycin resistance gene. Two hundred G418 resistant colonies were selected and analyzed for the expression of pp60c-src. Of the 50 cell clones found to have decreased levels of pp60c-src expression, three independent clones, which upon induction with Cd++ transcribed the expected 3.6 kb src complementary RNA, were further analyzed. In the presence of Cd++, these clones grew significantly slower than either the FR 18-1 parent or FR 18-1 cells transfected with the neomycin resistance gene alone. The morphology of these clones in the presence of Cd++ was distinct from that of either the parental FR 18-1 cells or normal FR3T3 cells. Additionally, the clones expressing the complementary src RNA were found to grow at reduced rates in soft agar, form fewer foci on monolayers of normal rat cells, and form

tumors more slowly following injection into syngenic rats when compared to parental FR 18-1 cells. These studies demonstrated that the level of pp60c-src kinase activity affects the growth characteristics and transformation properties of polyomavirus transformed cells.

We examined the effect of DNA tumor virus transformation of primary hamster embryo cells on the tyrosyl kinase activity of pp60c-src. Our present study demonstrates that some clones of hamster embryo cells transformed by simian virus 40, adenovirus 12, or bovine papillomavirus type 1 can possess elevated pp60c-src kinase activity when compared to normal hamster embryo cells. Other clones of hamster embryo cells transformed by these viruses were found to have normal levels of pp60c-src kinase activity. In those clones of transformed cells where pp60c-src kinase activity was found to be elevated, the increased levels of pp60c-src kinase activity was the result of an apparent increase in the specific activity of the pp60c-src phosphotransferase rather than an increase in the level of the src gene product. Additionally, pp60c-src was not found to be physically associated with tumor antigens known to be encoded by these viruses. These results showed that pp60c-src kinase activation can occur in hamster embryo cells transformed by several different DNA viruses and suggested that the molecular mechanism by which pp60c-src kinase activity is elevated may differ from that previously observed in polyomavirus transformed cells. These results also implied that elevation of pp60c-src kinase activity is not required for the transformation of hamster cells by these viruses.

A subpopulation of polyomavirus middle tumor antigens is capable of binding to cellular DNA. We examined the binding of Py early proteins to DNA cellulose. Our results showed that a small portion of MTag bound to both native and denatured calf thymus DNA cellulose under stringent binding conditions but did not bind under any conditions to Py DNA. The subpopulation of MTag that binds to DNA was found to be exclusive of the population of MTag that associates with pp60c-src. The results demonstrated that within Py-infected and -transformed rodent cells, MTag consists of a heterogeneous population that differs in potential for interactions with DNA and cellular proteins.

The pp60c-src molecules associated with MTag in Py transformed cells possess an additional post-translational modification not found on pp60c-src molecules not associated with MTag. This modification was determined to be a unique tyrosine phosphorylation site within the first 180 amino acids of the protein. This additional phosphorylation site is characteristic of pp60c-src molecules with increased levels of protein kinase activity in Py-transformed cells.

Polyomavirus MTag can be produced in large quantities using Py-transformed cells grown in nude mice. A recombinant plasmid containing a metallothionein promoter upstream of a Py MTag cDNA was constructed and used to transfect NIH 3T3 cells. Transformed cells injected into nude mice produced tumors within 3 weeks containing the level of MTag equivalent to that in 250 to 1,000 100 mm dishes of Py-infected cells.

Publications:

Amini, S., DeSeau, V. A., Reddy, S., Shalloway, D., and Bolen, J. B.: Regulation of pp60c-src by inducible RNA complementary to c-src mRNA in polyoma transformed rat cells. Mol. Cell. Biol. (In Press)

Amini, S., Lewis, A. M., Israel, M. A., Butel, J. S., and Bolen, J. B.: Analysis of pp60c-src protein kinase activity in hamster embryo cells transformed by simian virus 40, human adneoviruses, and bovine papilloma virus 1. J. Virol. 57: 357-361, 1986.

Bolen, J. B., Carey, K., Scheller, A., Basilico, C., Israel, M. A., and Prives, C.: A subclass of polyomavirus middle tumor antigen binds to DNA cellulose. J. Virol. 58: 157-164, 1986.

Kaplan, D. R., Bockus, B., Roberts, T. M., Bolen, J. B., Israel, M. A., and Schaffhausen, B. S.: Large scale production of polyoma middle tumor antigen using genetically engineered tumors. Mol. Cell, Biol. 5: 1795-1799, 1985.

Yonemoto, W., Bolen, J. B., Israel, M. A., Lipsich, L. and Brugge, J. S.: Use of monoclonal antibodies to probe the functional activity of the cellular src gene product in polyoma virus transformed cells. In Resifeld, S. and Sell, S. (Eds.): Monoclonal Antibodies and Cancer Therapy. New York, Alan R. Liss, Inc., 1985, pp. 551-564.

Yonemoto, W., Jarvis-Morar, M., Brugge, J. S., Bolen, J. B. and Israel, M. A.: Novel tyrosine phosphorylation within aminoterminal domain of pp60c-src molecules associated with polyoma virus middle tumor antigen. Proc. Natl. Acad. Sci., U.S.A. 82: 4568-4572, 1985.

Patents:

None

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01CP05481-01-LTVB

PERIOD COVERED

October 1, 1985 to September 30, 1986

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Biochemical Regulation of pp60c-src Protein Kinase Activity in Human Tumors

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	J. B. Bolen	Senior Staff Fellow	LTVB NCI
Others:	S. Amini	Visiting Fellow	LTVB NCI
	V. DeSeau	Biologist	LTVB NCI
	N. Rosen	Senior Investigator	MB NCI

COOPERATING UNITS (if any)

Department of Pathology, The George Washington University Medical Center,
Washington, D. C. (A. M. Schwartz)

LAB/BRANCH

Laboratory of Tumor Virus Biology

SECTION

Cellular Regulation and Transformation Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

1.5

PROFESSIONAL:

1.0

OTHER:

0.5

CHECK APPROPRIATE BOX(ES)

- (a) Human subjects (b) Human tissues (c) Neither
 (a1) Minors
 (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The protein products of proto-oncogenes possess functions (e.g., enzymatic activity) that play important roles in the cellular biochemical pathways which regulate normal cell growth and differentiation. While little is known about the biochemical functions of most proto-oncogene products, several are known to possess tyrosine-specific protein kinase activity. The most extensively studied of these tyrosine-specific protein kinases is pp60c-src. The product of the c-src proto-oncogene, pp60c-src, is the normal cellular homologue of the Rous sarcoma virus transforming gene, v-src, which encodes a closely related protein, pp60v-src. Both the v-src and c-src gene products are membrane-associated phosphoproteins which possess endogenous tyrosine-specific protein kinase activity. The transforming potential of pp60v-src and mutants of c-src appears to be related, in part, to the elevated specific activity of the v-src and mutated c-src encoded phosphotransferases. We have examined a variety of human tumor cell lines, human tumors and normal human tissues for pp60c-src tyrosyl protein kinase activity to determine the distribution and level of this enzymatic activity. The results of our studies demonstrate that pp60c-src protein kinase activity is significantly elevated in several apparently unrelated human cancers.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

J. B. Bolen	Senior Staff Fellow	LTVB	NCI
S. Amini	Visiting Fellow	LTVB	NCI
V. DeSeau	Biologist	LTVB	NCI
N. Rosen	Senior Investigator	MB	NCI

Objectives:

1. Analysis of pp60c-src kinase activity in human tumors.
2. Mechanism of regulation of pp60c-src kinase activity in human tumors.

Methods Employed:

1. Standard recombinant DNA technology for the cloning and propagation of Py chimeric plasmids.
2. Cell culture.
3. Cloning of mammalian cells.
4. Transfer of DNA into mammalian cells.
5. Transcriptional analyses of RNA including Northern and S1 analysis.
6. Immunoprecipitation analysis and protein kinase assays.
7. One and two-dimensional peptide analysis.
8. Two-dimensional phosphoamino acid analysis.
9. Immunoblot analysis.
10. High and medium pressure liquid chromatography of proteins and amino acids.
11. One and two-dimensional gel electrophoresis.
12. In vitro transcription and translation of cellular and viral mRNAs.
13. Generation of peptide-specific and amino acid-specific antisera.

Major Findings:

We observed a 20- to 40-fold increase in pp60c-src tyrosine protein kinase activity in human neuroblastoma cell lines over that observed in either human glioblastoma cell lines or human fibroblasts. The level of c-src gene transcripts and pp60c-src protein synthesis in the neuroblastoma cells was not significantly elevated when compared to the levels found in glioblastoma cells. Approximately one-half of the pp60c-src molecules synthesized during a 4h ³⁵S-methionine or ³²P-orthophosphate labeling period in neuroblastoma cells were found to migrate more slowly on SDS polyacrylamide gels than pp60c-src molecules labeled in glioblastoma cells. Peptide and phosphoamino acid analysis of the in vivo phosphorylated c-src molecules from these two cell types revealed that pp60c-src molecules from the neuroblastoma cells possess, in the amino-terminal portion of the protein, at least one unique tyrosine phosphorylation site not found in pp60c-src derived from glioblastoma cells.

We evaluated the level of pp60c-src protein kinase activity in a variety of human tumor tissues and human tumor cell lines and have estimated the abundance of the c-src protein in several of these tissues and cell lines. All cell lines

derived from tumors of neuroectodermal origin that express a neural phenotype were found to possess c-src molecules with high levels of tyrosine-specific protein kinase activity. In contrast, cell lines derived from tumors of neuroectodermal origin that do not express neural characteristics, such as glioblastomas and melanomas, were found to have pp60c-src molecules with low levels of protein kinase activity. A similar pattern was observed when we analyzed the activity of c-src molecules extracted directly from corresponding tumor tissues. Analysis of human tumor cell lines derived from tissues other than those of neuroectodermal origin revealed that pp60c-src protein kinase activity was low in most cases. Exceptions to this observation were all rhabdomyosarcoma, osteogenic sarcoma, Ewing's sarcoma, and colon carcinoma lines tested. Comparison of pp60c-src kinase activity in normal skeletal muscle and rhabdomyosarcoma tissue and in normal breast tissue and breast adenocarcinoma tissue revealed that pp60c-src kinase activity was specifically elevated in the tumor tissues in both cases. However, the amount of pp60c-src protein in both normal and tumor tissues was found to be similar. These observations suggested that increases in the specific activity of the pp60c-src phosphotransferase in some rhabdomyosarcomas and breast carcinomas may be a characteristic acquired during the malignant transformation of the cells that is retained in cell lines established from these tumors.

We found that pp60c-src molecules derived from cultures of human neuroblastoma cells have markedly elevated protein kinase activity when compared with pp60c-src molecules from either human glioblastoma cells or normal human fibroblasts. The elevation in protein kinase activity of pp60c-src molecules from neuroblastoma cells is not associated with increased pp60c-src abundance or due to stable association with other cellular proteins. The pp60c-src molecules synthesized in neuroblastoma cells possess decreased mobilities on SDS polyacrylamide gels when compared with the c-src protein synthesized in glioblastoma cells. The biophysical basis for the different electrophoretic behavior of neuroblastoma pp60c-src was localized to alterations within the amino-terminal half of the protein. Cell-free translation of cytoplasmic RNA from neuroblastoma and glioblastoma cells followed by immunoprecipitation and immune-complex kinase assays of the *in vitro*-synthesized proteins revealed that the resulting c-src molecules possessed similar mobilities on SDS polyacrylamide gels and had similar highly active protein kinase activities. The relationship between dendrite development and pp60c-src protein kinase activity was evaluated following retinoic acid-induced differentiation of neuroblastoma cells in culture. These experiments revealed that pp60c-src kinase activity increases as a function of dendrite production. These results suggest that the specific activity of pp60c-src molecules synthesized in human glioblastoma cells is negatively regulated by post-translational mechanisms which are less effective in regulating the activity of pp60c-src synthesized in human neuroblastoma cells. Our results imply that the degree of negative regulation of pp60c-src kinase activity in human neuroblastoma cells can be further diminished as a function of neural differentiation. (A manuscript describing these results entitled "Post-translational regulation of the *in vitro* protein kinase activity of pp60c-src from human neuroblastoma and glioblastoma cells" by Rosen, N., Amini, S., and Bolen, J. B. has been submitted for publication to the Journal of Cellular Biochemistry).

Publications:

Bolen, J. B., Rosen, N., and Israel, M. A.: Increased pp60c-src tyrosyl kinase activity in human neuroblastomas is associated with amino-terminal tyrosine phosphorylation of the src gene product. Proc. Natl. Acad. Sci. 82: 7275-7299, 1985.

Rosen, N., Bolen, J. B., Schwartz, A. M., Cohen, P., DeSeau, V., and Israel, M. A.: Analysis of pp60c-src protein kinase activity in human tumor cell lines and tissues. J. Biol. Chem. (In Press)

Patents:

None

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01CP05482-01-LTVB

PERIOD COVERED

October 1, 1985 to September 30, 1986

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Control of Papillomavirus Late Transcription

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	C. C. Baker	Medical Officer	LTVB	NCI
Others:	P. M. Howley	Chief	LTVB	NCI
	U. Linz	Visiting Fellow	LTVB	NCI
	J. S. Noe	Biologist	LTVB	NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Tumor Virus Biology

SECTION

Viral Oncology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

2.6

PROFESSIONAL:

1.6

OTHER:

1.0

CHECK APPROPRIATE BOX(ES)

- (a) Human subjects (b) Human tissues (c) Neither
- (a1) Minors
- (a2) Interviews

SUMMARY OF WORK (Use standard unabbreviated type. Do not exceed the space provided.)

The papillomaviruses cause benign and malignant lesions of squamous epithelia in higher vertebrates. The complete lytic cycle of these viruses (including late gene expression) occurs only in the differentiated cells of the squamous epithelium. Malignant lesions and infected cells in culture do not produce virus. An understanding of the transcriptional regulation of the papillomaviruses and its relationship to the control of epithelial cell differentiation is necessary for the elucidation of the role of the papillomaviruses in carcinogenesis. We have used bovine papillomavirus type 1 (BPV-1) as a model system for the study of late transcription and its control. Since there are no tissue culture systems which support late transcription, it has been necessary to analyze transcription occurring in the bovine fibropapilloma itself. A full length cDNA library has been constructed from mRNA isolated from bovine fibropapilloma tissue and has yielded several BPV-1-specific cDNAs not identified in a BPV-1-transformed C127 cell library. Of particular interest is that these mRNAs appear to be transcribed from a papilloma-specific promoter. This has been confirmed by primer extension and nuclease S1 analysis. The "late" or papilloma-specific promoter appears to be as much as 100-fold more active than the promoters used for transcription in BPV-1-transformed cells. We are currently attempting to identify the *cis* and *trans*-acting elements which are involved in the control of the late promoter and to determine the role which these *trans*-acting factors may play in epithelial cell differentiation. A second level of control of late transcription also occurs. Preliminary analysis of transcription occurring in nuclei isolated from BPV-1-transformed cells shows that transcription terminates in the late gene region upstream of the late polyadenylation site, effectively blocking the synthesis of late mRNAs. We are currently attempting to determine what sequences are necessary for transcription termination and to identify any factors which interact in trans with these sequences.

Project DescriptionNames, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

C. C. Baker	Medical Staff Fellow	LTVB	NCI
P. M. Howley	Chief	LTVB	NCI
U. Linz	Guest Researcher	LTVB	NCI
J. Noe	Biologist	LTVB	NCI

Objectives:

1. To study the control of late transcription of papillomaviruses using bovine papillomavirus type 1 (BPV-1) as a model system.
2. To analyze the structure and coding potential of viral mRNAs synthesized during productive infection by BPV-1.
3. To identify and assess the activity of the transcriptional promoters used in productively and nonproductively infected cells.
4. To determine the viral and/or cellular factors involved in the trans-activation of the major "late" viral transcriptional promoter.
5. To identify the cis-acting sequence elements involved in the control of the major "late" promoter.
6. To determine if transcription termination is an important mechanism of control of late transcription and to identify the cis-acting sequence elements involved in transcription termination.
7. To identify the viral and/or cellular trans-acting factors which control transcription termination.

Methods Employed:

1. Tissue culture for the preparation of viral mRNA from nonproductively infected cells.
2. Inoculation of calves by BPV-1 to generate fibropapillomas for the isolation of mRNA and factors produced during productive infection.
3. Standard isolation of DNA and RNA from cells and tissue.
4. Transcriptional analysis by cDNA cloning, Northern blotting, primer extension nuclease S1 protection and nuclear run-off analysis.
5. DNA sequencing of cDNA clones.
6. Functional analysis in vivo of transcriptional control elements by the transfer of recombinant plasmids into cells and the assay in vitro for recombinant gene products.
7. Purification of viral and/or cellular transcriptional regulatory factors from bovine fibropapillomas and BPV-1 transformed cells.
8. Functional analysis of transcriptional promoter sequence elements and trans-acting factors by in vitro transcription run-off analysis.

Major Findings:

1. A cDNA library has been constructed in the Okayama-Berg expression vector from mRNA isolated from a BPV-1 fibropapilloma. Six distinct classes of cDNAs have been analyzed. Five of the six cDNAs have 5' ends mapping near nucleotide 7250, indicating the presence of a major fibropapilloma promoter not previously identified in the analyses of the BPV-1-transformed cells. Transcription in

the fibropapilloma uses the transformed cell polyadenylation site at nucleotide 4200 as well as a fibropapilloma-specific polyadenylation site at nucleotide 7175. These cDNAs potentially encode the L1 (major capsid), L2 (minor capsid), c-terminal E2, and a fibropapilloma-specific E4 proteins. The E4 cDNA is the most abundant species present in the fibropapilloma cDNA library, consistent with the data of Gallimore showing that the HPV-1a E4 protein is a very abundant cytoplasmic protein in HPV-1a warts. A manuscript describing this library is in preparation.

2. The 5' termini of mRNAs from bovine fibropapillomas and BPV-1-transformed C127 cells have been analyzed using the primer extension and nuclease S1 protection methods to map the promoters and determine their relative activity in these different tissues and cells. The most active promoter in the fibropapilloma has been mapped to near nucleotide 7250. This promoter is not active in the BPV-1-transformed C127 cell and most likely represents a productive infection-specific promoter. The upstream sequences include a tandem repeat of sequences homologous to the SV40 late promoter sequence, GGTACCTAACC, as well as a CCAAT box. An additional promoter in this region appears to be active in both the fibropapilloma and the transformed cells and has an RNA start site at nucleotide 7185. This promoter overlaps the late mRNA polyadenylation site and indeed appears to use the AATAAA polyadenylation signal as a TATA box. Another previously unrecognized promoter has a heterogeneous RNA start site near nucleotide 7940 and is active in both the fibropapilloma and transformed cell. The sequences upstream of this start site contain a TATA box, CCAAT box and three potential SP1 binding sites. The previously identified promoters with RNA start sites at nucleotides 89, 2443 and 3080 have been confirmed and have been shown to be active in both the fibropapilloma and transformed cell. In addition, Barbara Spalholz (LTVB) has shown that the nucleotide 7940 and 89 promoters can be trans-activated by the viral E2 product. A manuscript describing these results is in preparation.

3. Transcriptional mapping in in vitro nuclei isolated from BPV-1-transformed cells has demonstrated that although no "late" mRNA is made in the transformed cell, transcription does proceed through most of the "late" region. Transcription appears to terminate near the 3' end of the late region, but upstream of the late polyadenylation site. Thus, transcription termination may be a major cause of the block in late mRNA synthesis and virus production in the transformed mouse cell. To further study transcription termination in the late region, we have cloned the BPV-1 XbaI to HindIII fragment (nucleotides 6134 to 6959) into the PSV2CAT expression vector upstream of the polyadenylation site of the vector. The presence of this fragment reduces CAT activity in a transient expression assay when cloned in the forward, but not the backward, orientation with respect to the CAT transcription unit, similar to what has been shown for the beta-globin transcription termination region. Progressive deletions will be introduced into this plasmid to further map the transcription termination sequence.

Publications:

None

Patents:

None

ANNUAL REPORT OF

THE LABORATORY OF VIRAL CARCINOGENESIS
BIOLOGICAL CARCINOGENESIS PROGRAM
DIVISION OF CANCER ETIOLOGY
NATIONAL CANCER INSTITUTE

October 1, 1985 through September 30, 1986

FUNCTIONAL STATEMENT: The Laboratory of Viral Carcinogenesis (LVC) has been charged with the planning, development, implementation, and coordination of research programs on the etiology and mechanisms of carcinogenesis. In the past, particular emphasis was placed on delineating the roles, mechanisms and regulation of action of oncogenic viruses, virus-related and virus-associated genetic sequences, and gene products. Research efforts were conducted on virus-host relationships in virus-induced cancers, focusing on the detection and characterization of oncogenic viruses, their mechanisms of genetic integration and expression, and their modes of transmission in animals and man. The rapid technological development of eukaryote genetics and molecular biology has resulted in a synthesis of viral and cellular gene action during neoplastic processes. As a result, the research emphasis of the Laboratory has converged on the elucidation of regulatory events which are operative in human cancers and homologous animal models. The multidisciplinary understanding of the neoplastic process, combining knowledge and technology from immunology, pathology, physiology, molecular biology, and genetics is the common character of the various research projects of the laboratory, with a constant consideration of therapeutic opportunities. Activities of the Laboratory are conducted by in-house research and collaborative agreements with other research organizations.

SCIENTIFIC BACKGROUND AND SIGNIFICANCE: The combination of technological advances, and a number of coordinate, empirical observations, has dramatically altered the thinking of the scientific community on the molecular and genetic mechanisms of human and animal carcinogenesis. The procedures of molecular cloning, DNA sequencing, hybridoma production and somatic cell genetics have pushed the study of eukaryote genetic analysis from a speculative and interpretive discipline to the level of viewing gene action directly. A revolution in biological thinking and analysis is upon us, and these methodologies are being applied as rigorously to the dissection of carcinogenesis as to any other biological process. The contributions to the generalized journals (i.e., Science, Nature, and Cell) have experienced a quantum increase in definitive studies on the mechanism of carcinogenesis and transformation.

Several major advances are responsible for our changes in thinking, and these concepts and developments have influenced, and in part been influenced by, the research effort of the LVC/NCI. Among these generalized advances are: (1) The development of the concepts and properties of vertebrate "oncogenes." These loci are normal cellular genes which were initially described as transduced RNA segments in transforming retrovirus genomes and have also been discovered by focus induction after transfection of mouse 3T3 cells with genomic DNA extracted from human tumors. The limited number of proto-oncogenes (circa 40) described to date has attracted considerable research emphasis over the past few years as an experimental opportunity to study neoplastic transformation directly from both genetic and molecular perspectives. Because of these extensive analyses, there

are now at least five documented modes of oncogene activation associated with tumorigenesis. These include: (a) transduction of proto-oncogene transcripts by retroviruses, thereby placing the oncogene under regulatory control of strong promoters in the viral long terminal repeat (LTR); (b) chromosomal insertion of an infecting retrovirus adjacent to a proto-oncogene similarly altering their control of transcription; (c) translocation of cellular oncogenes to chromosomal regions of differential regulation; (d) amplification of oncogene-containing segments, thereby increasing the dosage of the oncogene; and (e) point mutation in the cognate cellular oncogene. The dramatic demonstration that the human "oncogenes" were, in many cases, homologs of the retroviral "oncogenes" which have been studied for decades, unified these formerly disparate gene sets into a single group which immediately became a viable candidate for somatic cell targets of carcinogenic insults. (2) The normal functional role of these oncogenes in normal (or in neoplastic) tissues was, for sometime, obscure. Within the last few years, by sequence alignment of cloned DNAs of various oncogenes, three putative matches have revealed a functional association of oncogene products. Specifically, the sis oncogene encodes platelet-derived growth factor; the erbB oncogene encodes the receptor for epidermal growth factor and the transferrin receptor is functionally related to the ras oncogene product. In addition, the fms product has recently been shown to encode the cellular receptor for macrophage colony stimulating factor. The functional relationship between the remaining 30+ oncogenes thus far described is an area of intense research efforts in many laboratories to date. (3) Virtually all of the human oncogene homologs have now been chromosomally mapped to specific positions on the human gene map. These are in addition to the nearly 1500 different loci comprising the human genetic map. In addition to the proto-oncogene loci, approximately 20 additional described loci are thought to participate in neoplastic transformation in man (e.g., growth factors, cell surface antigens, retroviral receptors, and integration sites, etc.). A dramatic advance has been the demonstration that certain human tumors with specific chromosomal rearrangements can be interpreted to involve the modulated regulation of cellular oncogenes by normally distant DNA regulatory elements which have been placed adjacent to the oncogenes by chromosomal rearrangement. (4) Despite a long history of unsuccessful attempts to isolate human type C retroviruses in a valiant effort by the former Virus Cancer Program, only very recently have two human diseases been associated with exogenous human retroviruses. These are adult T-cell leukemia, which is etiologically associated with human T-cell leukemia virus-I and -II, and acquired immune deficiency syndrome (AIDS), which has been serologically correlated with the development of antibodies to a type D retrovirus designed HTLV-III or LAV. These isolates are so new that Koch's postulates are yet to be tested in animal models, yet the correlation data is compelling. (5) The study of animal models of certain of the homologous diseases in primates, rodents, and cats has provided terrific opportunities for reconstruction and analysis of the initiation and progress of mammalian tumors. For example, the AIDS models in primates (simian acquired immune deficiency syndrome [SAIDS]) and in FeLV-infected cats (feline acquired immune deficiency syndrome [FAIDS]) have modified significantly our interpretation of retroviral pathology to include broad levels of immune impairment in addition to (and possibly, in combination with) leukemogenesis.

The most significant findings of the LVC during this year are discussed below.

1. Identification of proto-oncogene fusion to generate oncogenes with two or three functional domains during tumorigenesis and during mammalian evolution.
The cumulative techniques of cell genetics, molecular biology, linkage analysis

and in situ hybridization has resulted in the identification and characterization of over 1500 human loci, a value which now exceeds the number of genes mapped in Drosophila. We have concentrated our efforts on somatic cell hybrid panels and in situ hybridizations to genes related to neoplastic processes including (1) cellular proto-oncogenes, (2) growth factors, (3) growth factors and receptors, (4) endogenous retroviral families, (5) integration sites for retroviruses, and (6) restriction genes that delimit retrovirus replication in mammals. Within the last few years, the human gene map has experienced a large increase in the number of neoplasia loci that have been mapped to specific chromosomal positions. Of the 35 specific human loci that have been chromosomally mapped to date, 13 (40%) have been assigned by the Genetics Section scientists and their collaborators. This year, we have concentrated on understanding the genomic organization of several genes: rel, ets, trk, tpr, fms, met and endogenous retroviral families. Three of these genes, ets, met-tpr and trk, were found to be composite genes derived from the fusion of chromosomally disparate functional loci. Truncation of these cellular genes in a variety of human neoplasias, as well as in certain non-neoplastic pathologies (e.g., ets-2 in Down's syndrome or met in cystic fibrosis) which were suggested by their chromosomal position are under investigation. The emerging gene map neoplasia-associated loci continues to provide an unprecedented opportunity for molecular genetic analysis of the initiation and progression of neoplastic processes.

2. Demonstration of non-specific chromosomal dispersal of endogenous human retroviral families and exogenous HTLV-I in de novo human tumors. T-cell lymphotropic retrovirus (HTLV-I, II and III) have an affinity of human T lymphocytes and enter the cells by specific interaction with the T-4 receptor. All three types of HTLVs have been transmitted in vitro, molecularly cloned and sequenced. Despite these advances, the mechanism by which infection with these viruses results in malignant transformation or immunosuppression remains unknown. We are focusing on basic mechanisms, both on a cellular and molecular level, by which these viruses transform or immunosuppress. In order to address whether HTLV-I may induce transformation through an insertional mutagenesis mechanism, we have utilized somatic cell hybrids constructed between rodent cells and HTLV-I infected human cell lines to study the processes and consequences of HTLV chromosomal integration. Integration in vitro was shown to be a dynamic process and proviral integration apparently occurs at random in the genome. We have also utilized the panel of Hut 102 X Chinese hamster hybrids to demonstrate that the novel Class I antigenic determinants expressed on HTLV-I infected cells do not result from induction of Class I genes encoded by the cellular MHC locus, but are probably encoded by integrated HTLV-I. The activities of the promoter unit contained within the LTR of both HTLV-I and HTLV-III were examined by transfecting various cells with recombinant plasmids containing the LTR of HTLV-I or HTLV-III linked to the bacterial gene for chloramphenicol acetyltransferase (CAT). We have demonstrated that infected cells contain factors that act in trans on the LTRs of the infecting virus to activate transcription. Two families of endogenous retroviral sequences were shown to be widely dispersed in the human genome using genetic analysis. The sequences are the consequence of an ancient evolutionary gene amplification which resulted in greater than 0.1% being homologous to retroviral sequences.

3. Demonstration of widespread occurrence of antibodies to HTLV-III in pooled human immunoglobulin and in recipient patients. Immunoglobulin produced from large pools of plasma has been used successfully to protect susceptible patients from infections by providing passive immunity. We have tested 63 sera obtained

from 23 patients with primary immunodeficiency syndromes between 1980 and 1986, before and immediately after immunoglobulin infusion. Using Western immunoblots, we found 52 sera to be HTLV-III/LAV antibody positive. There was a clear correlation between the amount of antibody present after infusion and the titer of antibody in the immunoglobulin lot administered. Since these patients cannot produce antibody, the data are consistent with an exogenous origin of the antibody and suggest that a positive titer for HTLV-III antibody in such patients is not the result of active infection with AIDS virus.

4. Molecular and functional dissection of regulatory elements in the genome of bovine leukemia virus and their homology to controlling units in HTLV. Bovine leukemia virus (BLV) and the human T-cell leukemia viruses (HTLV-I and HTLV-II) constitute a unique subgroup of RNA tumor viruses; they share a similar genetic organization, promoter architecture, and common strategy for regulating gene expression. As a group, these viruses are generally quiescent in their host; however, infected lymphocytes often become virus producers when transferred to in vitro culture conditions. Moreover, cell lines have been infected in vitro and often become virus positive. It was previously shown that the BLV promoter unit, contained in the proviral LTR, was transcriptionally inert in all cell lines except those which were productively infected with BLV. The cis-acting sequences in the LTR (adjacent to the promoter) which were required for transcriptional activity were defined by deletion mapping and by construction of chimeric promoter units. The CAT system was used to monitor gene expression and promoter activity. The BLV LTR contained sequence elements both upstream and downstream of the RNA start site that were required for optimal gene expression. The upstream element behaved like a condition-specific enhancer element (or response element, see below) which was active only in cells productively infected with BLV. The downstream sequence element was found to increase gene expression when located 3' from the RNA start site of several promoter units and in all cell types, irrespective of BLV infection. BLV, like HTLV-I and HTLV-II, possesses several open reading frames in the pX region, located between the envelope gene and the 3' LTR. To determine whether the expression of the BLV pX genes results in transcriptional transactivation of their LTRs, uninfected cells were co-transfected with a plasmid containing the CAT gene controlled by the BLV LTR and expression plasmids containing BLV pX coding sequences. Synthesis of CAT enzyme, directed by the BLV LTR, is detected only when an active pX is present in target cells reflecting the transactivation induced or produced by pX genes. Although the mature, spliced pX mRNA has the potential to code for at least two proteins of ca. 38,000 and 17,000 MW, it was found that expression of the larger MW protein is necessary and sufficient to produce the transactivation effect. To define the sequence elements in the LTR that respond to the immediate effects of pX expression, uninfected cells were co-transfected with a BLV pX expression plasmid and plasmids containing the CAT gene controlled by portions of the BLV LTR or chimeric promoters containing both BLV and SV40 promoter elements. By in vitro gene swapping experiments, the target of pX action in the LTR was found to be composed of at least two pX response elements. The first is located within a region between 100bp and 170bp upstream of the RNA start site, and another is located immediately upstream of the TATA box. It is interesting to note that both HTLV-I and HTLV-II LTRs possess several short repeated sequences that, while not homologous to the BLV sequences, may play an analogous role in transcriptional regulation.

5. Expansion of the human raf proto-oncogene family to four chromosomally distinct loci and evidence for transcriptional modulation in certain human neoplasias. The major advances in recent studies on raf oncogenes were: (1) Isolation, sequence determination, chromosomal mapping and characterization of biological activation of novel raf genes, A-raf-1 from mouse and man and A-raf-2 from man. This increases the number of human raf-related genes to four, two of which, c-raf-1 and A-raf-1, are active genes. All of these genes are relevant to human pathology, c-raf-1 and A-raf-1 because their product has transforming activity, and c-raf-1 is implicated in a variety of human carcinoma on cytogenetic and biochemical grounds. C-raf-2, located near the top of the short arm of chromosome 4, has several restriction enzyme polymorphisms associated with its flanking DNA and, thus, has become a useful clinical marker for a hereditary disease, Huntington's Chorea. A-raf-2, while more distant than another active oncogene met to the site of the cystic fibrosis locus on chromosome 7, may still be useful in further characterizing this region. (2) Experiments to functionally map c-raf-1 and A-raf-1 products in the signal transduction pathway of growth factors have shown that both genes appear to act downstream of ras oncogenes, making them the end of a chain which includes most other peripheral cytoplasmic and membrane-associated oncogenes. Thus, raf genes and their regulation would appear to be ideal targets for the tailoring of modulating drugs with a potential for therapeutic significance. (3) The c-raf-protein associated ser/thr specific kinase was further characterized. N-terminal truncation appears to increase kinase activity, as well as transforming ability. (4) There is a division of labor between members of the raf family, c-raf-1 appears to have a basic regulatory role in most tissues, i.e., it is expressed everywhere, albeit at varying levels, whereas A-raf-1 is highly restricted in its expression with highest levels in the epididymis. (5) Biochemical evidence was obtained for a role of c-raf-1 in all histological types of lung carcinoma. The gene is expressed at unusually high levels in 60-80% of all tumors as determined by Northern, immunoblot and immunohistochemical techniques. We are currently evaluating the role of c-raf in transformation of these cells. To aid in this evaluation, an animal model system was developed for rapid (5-13 weeks), high frequency induction of lung adenocarcinoma in mice. The tumors contain transforming DNA, as judged by DNA transfection, and express uniformly high levels of c-raf RNA and protein. In fact, the levels are well in excess of those seen in NIH 3T3 cells transformed by an LTR-driven c-raf-1 cDNA construct. Live cell fluorescence indicated surface fluorescence of the predominantly cytoplasmic c-raf protein. Attempts to modulate tumor induction and promotion in these mice by stimulation of an anti-raf protein-directed immune response yielded promising results since the latency, but not the final evidence of tumor development in mice promoted with BHT, was almost doubled.

6. Biological and molecular genetic dissection of the synergistic action of raf and myc oncogenes in tumorigenesis. (1) v-raf and v-myc oncogenes act synergistically in vivo in the mouse for induction of carcinoma, as well as lymphoid/hematopoietic tumors and in vitro in the transformation of macrophages, myeloid stem cells, and B cells from primary hematopoietic stem cell cultures. The basis for this synergism is a combination of signals required early (competence) and late (progression) in the G1 phase of the cell cycle. (2) High level expression of v-myc can abrogate the requirement of hematopoietic/lymphoid and fibroblastic cells for a variety of c-myc or, more generally, competence gene-inducing growth factors including IL-3, IL-2, and PDGF. Thus, myc appears to serve as a second messenger function for competence-inducing growth factors. (3) Evidence for an autoregulatory mechanism in c-myc

transcription regulation was established in three different lineages of mouse cells infected with recombinant retroviruses expressing high levels of v-myc. High level expression of the full complement of v-myc sequences invariably shut off c-myc expression in these cells and analysis of v-myc deletion mutants has defined elements within v-myc controlling nuclear localization, and c-myc suppression and induction. Moreover, we have extended this observation to include N-myc in the cross-regulation of c-myc expression.

7. Identification of potentially diagnostic transforming growth factors in concentrated urine from breast cancer patients. Procedures for concentration and initial separation of TGF-alpha, EGF, and TGF-beta from human urine were developed using adsorption to microparticulate silica and fractional elution with increasing concentrations of acetonitrile. Further resolution was obtained by sequential chromatography based on molecular size (Bio-Gel), charge (CM-cellulose), and hydrophobicity (RP-HPLC). The high molecular weight (HMW) of 30,000 to 35,000 Mr for TGF, previously reported in the urine of various cancer patients, is, in patients with malignant astrocytomas, indistinguishable from the HMW form of hEGF in terms of apparent molecular size, EGF receptor binding activity, EGF immunoreactivity and clonogenic activity. However, in comparison to bulk (25 liters) urine from normal individuals, equivalently large urine samples from these brain tumor patients contained about fourfold more HMW hTGF/hEGF. hTGF-alpha was not identified in either source of bulk urine. The urine of patients with disseminated breast cancer contains immunoreactive TGF-alpha which is not present in comparable control urine from normal individuals. Currently, the pre- and postoperative urine of patients with primary breast cancer without metastasis is being compared for TGF-alpha activity.

8. Specific identification and molecular characterization of cellular genes that specify susceptibility to tumor promoter-induced neoplastic transformation in mice and humans. Evidence for the involvement of such genes in animal and human systems has come from the observations that there are irreversible steps in tumor promotion and that animals can be bred for sensitivity to tumor promotion. Two genes that specify sensitivity to promotion of neoplastic transformation by tumor promoters in mouse epidermal JB6 cells have been cloned. These putative genes, termed pro-1 and pro-2 have been sequenced and are being characterized with respect to mode of activation and regulation of expression. The sequence of pro-1 is intronless, shows the consensus sequences needed for transcription and translation, and is expected to code for a 7000 dalton protein. Tumor promoter treatment of P⁺ cells produces transient increases in pro-1 RNA levels. A lesser increase in pro-1 RNA was seen in TPA-treated P⁻ cells. Human nasopharyngeal carcinoma cell (CNE) DNA confers promotion sensitivity (P⁺ activity) on resistant JB6 mouse cells. This activity is, at least in part, attributable to activated homologs of mouse pro-1, as shown by constructing a genomic library of CNE₂ DNA, screening with a mouse pro-1 probe, and testing the homologs for P⁺ activity after transfection into resistant mouse cells. The pro-2 homologs isolated from the library proved to be inactive. Inactive pro-1 homologs isolated from a normal human library and from the CNE₂ library are being compared with activated CNE₂ pro-1 to ascertain the mode of activation. Two cDNA libraries, one from initiation-promotion induced skin papillomas and the other from a squamous carcinoma, have yielded clones homologous to pro-2 each containing a 2.1-kb cDNA. This finding is significant in that it not only facilitates intron-exon assignment in the genomic clone, but also opens up investigation of the role of pro-2 expression in carcinogenesis *in vivo*. Finally, a novel transforming activity has been detected by transfecting the DNA of either TPA transformed P⁺

cells or DNA of CNE cells into JB6 P⁺ recipients. It is postulated that pro gene expression triggers the constitutive expression of these novel transforming genes.

9. Implication of phorbol ester receptor protein kinase C (PKC) in signal transduction of TPA mediated tumor promotion. The goal of the signal transduction project is to determine the required biochemical events occurring between the tumor promoter receptor interaction and the activation of nuclear effectors of neoplastic transformation. Candidate second messengers include protein phosphorylation, reactive oxygen generation, and calcium mobilization. Both activation of the phorbol ester receptor, PKC, and the subsequent loss of PKC activity may be on the signal transduction pathway for TPA-promoted transformation. Some 16 C kinase substrates have been identified in JB6 cells. One such substrate, the 80-kd heat shock protein (hsp 80), is TPA-inducible at the level of phosphorylation and possibly synthesis and its constitutive level is inversely related to the degree of preneoplastic progression. TPA-transformed tumorigenic cells show no detectable hsp 80. This PKC substrate may function as a suppressor of neoplastic transformation. Lanthanides, which are pharmacologic analogs of calcium, function as active promoters of neoplastic transformation in JB6 cells. Although lanthanides can activate PKC in cell free assays, they do not activate PKC in intact cells. The pathway of lanthanide-induced promotion may, however, intersect with phorbol ester-induced promotion at the level of a 23-kd PKC substrate, whose migration, and possibly biological activity, is altered by lanthanides. The lanthanides, like phorbol esters, induce transformation in activated pro-1 or pro-2 transfectants. This indicates that tumor promoters can collaborate with activated pro genes to bring about neoplastic transformation by either PKC-dependent or PKC-independent pathways. The synthesis of nuclear proteins of 15 and 16-kd is TPA inducible in P⁺ but not in P⁻ cells, an event that may account, in part, for the promotion sensitivity of P⁺ cells. Finally, P⁺ cells and P⁻ cells differ in a transient, TPA-stimulated focus-associated expression of cellular P21 H-ras and an irreversible change in actin configuration, suggesting a possible collaboration of cytoskeletal, cytoplasmic and nuclear proteins with pro genes to bring about transformation.

10. Development of the domestic cat as an animal model for genetic analysis with emphasis on proto-oncogenes, immune system genes and endogenous retroviruses. The development of the domestic cat as an animal model for genetic analysis began with the construction of a genetic map which now consists of over 50 loci. A striking linkage homology of the human and feline gene maps was confirmed by cytological procedures and today over 25% of the human genome can be aligned band for band with the feline karyotype. Typing sera for the feline major histocompatibility complex were generated by allogenic skin grafting and serological reagents were evaluated by population cluster analysis and by immunoprecipitation of feline lymphocyte antigens. The molecular organization of the feline MHC complex was derived using homologous gene clones and compared to the human and murine MHC clusters. Evolution of the proto-oncogene family of mammals has been studied by mapping these genes in cat, mouse and man, and reconstructing their natural history. Two endogenous retroviral families (RD-114 and FeLV) have been studied from a molecular perspective and appear to represent vestiges of ancient infections and gene amplification in the Felidae. An evolutionary tree of 37 species of the cat family has been constructed using two molecular measures of genetic distance.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE		PROJECT NUMBER	
NOTICE OF INTRAMURAL RESEARCH PROJECT		Z01CP05150-07 LVC	
PERIOD COVERED October 1, 1985 to September 30, 1986			
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Isolation of New Oncogenes			
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)			
PI:	Ulf R. Rapp	Chief, Viral Pathology Section	LVC NCI
Others:	John L. Cleveland	Staff Fellow	LVC NCI
	Takayasu Matsugi	Visiting Fellow	LVC NCI
COOPERATING UNITS (if any) Department of Pathology, University of Helsinki, Finland (J. Keski-Oja)			
LAB/BRANCH Laboratory of Viral Carcinogenesis			
SECTION Viral Pathology Section			
INSTITUTE AND LOCATION NCI, NIH, Frederick, Maryland 21701-1013			
TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:	
1.4	1.1	0.3	
CHECK APPROPRIATE BOX(ES)			
<input type="checkbox"/>	(a) Human subjects	<input type="checkbox"/>	(b) Human tissues
<input type="checkbox"/>	(a1) Minors	<input checked="" type="checkbox"/>	(c) Neither
<input type="checkbox"/>	(a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) Two approaches have been used by this laboratory for the isolation of new oncogenes: (1) transduction with retrovirus and (2) transformation of cells by retrovirus insertion followed by molecular cloning of flanking DNA. The first approach has yielded the <u>v-raf</u> oncogene-carrying virus 3611MSV and several other isolates which will be molecularly analyzed in the future. For the second approach an <u>in vitro</u> system was established for the transformation of rodent epithelial cells by C3H MuLV in conjunction with 12-O-tetradecanoylphorbol-13-acetate (TPA). Transformed cell clones obtained from soft agar generally were virus nonproducers. The purpose of these experiments was to sequence-label TPA-promotable cellular tumor genes, and thus, make possible their isolation by molecular cloning. Epithelial rodent cells that had been transformed with MuLV and TPA are being examined for expression of novel virus-cell hybrid transcripts and are being used for the molecular cloning of LTR-linked cellular DNA. As an example for the principle effectiveness of such a strategy, we have demonstrated for NIH 3T3 cells transformed by transfection with MuLV LTR DNA that the proto-oncogene, <u>c-raf-1</u> , was activated as an oncogene by a promoter insertion mechanism. Moreover, we have recently described, for <u>in vitro</u> -infected and transformed myeloid cells, integration of MuLV into the sixth exon of the <u>myb</u> proto-oncogene. The effect of integration was a truncation of the <u>myb</u> protein which may contribute to its transforming ability. Integration was precisely at the site previously identified for insertional activation of <u>c-myb in vivo</u> by another strain of MuLV.			

PROJECT DESCRIPTIONNames, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Ulf R. Rapp	Chief, Viral Pathology Section	LVC	NCI
John L. Cleveland	Staff Fellow	LVC	NCI
Takayasu Matsugi	Visiting Fellow	LVC	NCI

Objectives:

To obtain new oncogenes which might be involved in common human tumors; to develop improved in vitro systems for isolation of genes involved in chemical transformation of epithelial cells; and to isolate, using techniques of cocarcinogenesis, new cell-derived tumor genes after their linkage with or incorporation into type C viral genomes.

Methods Employed:

A basic goal of chemical carcinogenesis research is the identification of cellular genes which bring about the transformed phenotype of cells after carcinogen exposure. Assays for quantitative chemical carcinogenesis and cocarcinogenesis with cultured mouse cells have been widely accepted as model processes that occur during the development of spontaneous tumors. While this methodology has been highly successful in delineating details of the metabolic activation of carcinogens and the gradual transformation of cells by such agents and tumor promoters, it has not yielded much information regarding the genes and genetic mechanisms involved.

RNA tumor viruses, on the other hand, while not being serious candidates as causative agents for most human tumors, have provided the first isolates of cellular genes which are apparently sufficient to induce malignant transformation. To combine the advantages of both systems, experiments have been initiated to identify the genes involved in chemical transformation and the regulation of their expression in vitro. Efforts to identify such genes have included (1) screening of chemically transformed cells for the expression of known type C virus-transduced tumor genes, (2) isolation of additional transforming viruses from genes for screening, and (3) attempts to identify genes promotable by 12-O-tetradecanoylphorbol-13-acetate (TPA) by sequence-labeling with murine leukemia virus (MuLV) genomes followed by molecular cloning of genes that were constructed in these cells by LTR promoter insertion.

Most recently, an additional strategy evolved out of experiments with v-myc carrying retroviruses. Tumors induced by such viruses develop with kinetics that suggest secondary oncogene activation. To isolate the latter oncogenes, tumor DNA will be transfected onto v-myc expressing NIH 3T3 cells.

Major Findings:

The potential of activating proto-oncogenes to transforming genes by MuLV LTR promoter insertion has recently been demonstrated by us. In an attempt to

establish a novel strategy for the identification of potential cellular oncogenes (c-onc genes), malignant cell lines were isolated after co-transfection of normal NIH 3T3 (carrier) DNA and cloning of Moloney leukemia virus long terminal repeat (Mo-LTR) sequences onto NIH 3T3 recipient cells (Muller and Muller, 1984). Theoretically, such an approach can lead to the induction of neoplastic transformation in several ways, including (1) the transcriptional activation of neighboring genes, via the Mo-LTR promoter or enhancer; (2) blocking of gene transcription as a consequence of Mo-LTR integration; (3) transcriptional activation of carrier DNA sequences by juxtaposition to cellular enhancers or promoters (as suggested by Cooper et al., 1980); or (4) expression of structurally aberrant proteins from truncated rearranged or mutated carrier DNA sequences. The transforming gene of one LTR transformed clone (designed clone S1) was shown to be homologous to the murine retroviral oncogene *v-raf*, which in turn is homologous to one of the two oncogenes (*v-mil*) transduced by the avian leukemia virus MH2. The oncogenic activation of *c-raf* occurred by Mo-LTR integration into the fifth intron of the proto-oncogene leading to the synthesis of high levels of *c-raf* transcripts starting in the Mo-LTR. Transcriptional activation of *c-raf* is accompanied by the synthesis of large amounts of cytoplasmic *c-raf* protein. These findings indicate a promoter insertion mechanism of *c-raf* activation.

A second example of insertional oncogene activation by MuLV *in vitro* was established by us recently. Infection of fetal liver cells in culture in the presence of Interleukin 3 (IL3), followed by selection of transformed myeloid cells, yielded a clonal line in which MuLV (leuk strain) integrated into the sixth exon of *c-myb*. Curiously, the site of integration was identical on the level of nucleotides to a second insertion event that had occurred *in vivo* (in NFS 60 cells).

Use of *v-myc*-carrying murine retroviruses has recently been employed by us for generating mouse tumors in a variety of tissues. Because of the long latency of tumor development, we suspect that additional oncogenes became activated in these tumors. Transfection of tumor DNAs is currently ongoing, using *v-myc*-expressing NIH 3T3 target cells, for isolation of these additional oncogenes.

One *in vivo* system in which *v-myc*-carrying MuLV were used was the Balb/C-Pristane system for induction of plasmacytomas. Plasmacytomas can be induced in genetically-susceptible inbred strains of mice, such as BALB/c and by the intraperitoneal (i.p.) injection of pristane (2,6,10,14 tetramethylpentadecane). These tumors arise slowly, beginning at 120 days and with a mean latent period of 210 to 220 days. We discovered that plasmacytomas appear as early as 54 days after injection of 0.5 ml pristane if it is followed in 7 to 33 days by infection with a retroviral construct, J-3 and a Moloney murine leukemia helper virus. J-3 contains a defective *raf/mil* and a hybrid avian *v-myc* gene derived from the MH2 and MC29 viruses. Over 95% of pristane-induced plasmacytomas have chromosomal translocations involving chromosome 15 and an associated deregulation of *myc* gene transcription. In contrast, all but one of the plasmacytomas induced in mice by J-3 virus and pristane lack chr 15 translocations or evidence of *c-myc* gene rearrangement. J-3 appears to replace the requirement for chromosomal translocation in plasmacytomagenesis, presumably by directly supplying high levels of *v-myc* transcripts.

Publications:

Cleveland, J. L., Weinstein, Y., Rapp, U. R., Askew, D. and Ihle, J. N.:
Insertional mutagenesis in vitro in primary hematopoietic stem cell cultures.
Curr. Top. Microbiol. Immunol. (In Press)

Patents:

None

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01CP05180-06 LVC

PERIOD COVERED

October 1, 1985 to September 30, 1986

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Evolution and Sequence Organization of Mammalian Retroviruses

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Raoul E. Benveniste Medical Officer LVC NCI

Other: Stephen J. O'Brien Chief LVC NCI

COOPERATING UNITS (if any)

Yale University, New Haven, CT (C. Sibley)

LAB/BRANCH

Laboratory of Viral Carcinogenesis

SECTION

Viral Leukemia and Lymphoma Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701-1013

TOTAL MAN-YEARS:

0.9

PROFESSIONAL:

0.4

OTHER:

0.5

CHECK APPROPRIATE BOX(ES)

- (a) Human subjects (b) Human tissues (c) Neither
- (a1) Minors
- (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

We have previously demonstrated that retroviruses have been transferred, under natural conditions, between vertebrate species that are only remotely related phylogenetically. Some of these transfers have resulted in the subsequent incorporation of the retroviral genome into the germ cell DNA. Five examples of retroviral transfer have been documented, including a transfer from Old World primates to domestic cat ancestors, from New World primates to skunk ancestors, and from rodents to the ancestors of the domestic cat, pig, and mink. There are also two examples of virus transfer between sympatric species that have not resulted in incorporation of the acquired genome into the germ line. One case involves the transfer of endogenous viruses of Southeast Asian rodents to gibbons and the other is the transfer of endogenous retroviruses of langurs to macaques. The direction of retroviral transfer between species can be established by examining the cellular DNA of related species for viral sequences. In the New World primate (squirrel monkey) to skunk transfer, no sequences related to the squirrel monkey retrovirus (SMRV) were detected in mink and weasel cellular DNA. These two species are believed to be close relatives of the skunk. A phylogenetic tree of the carnivores was, therefore, derived from the thermal stability measurements of nonrepetitive cellular DNA; it differs in several respects from the classical relationships derived by anatomical considerations and the fossil record. The two species of skunks examined are accorded separate family status and shown not to be closely related to the Mustelids (minks and weasels).

The lentiviruses isolated from goats (CAEV), sheep (visna), horses (EIAV) and man and primates (HTLV-III/LAV, STLV-III, MnIV) are present only in infected individuals of those species. The origin of these viruses is currently being examined; the identification of the endogenous host species might permit control of the vectors for virus spread.

PROJECT DESCRIPTIONNames, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Raoul E. Benveniste	Medical Officer	LVC	NCI
Stephen J. O'Brien	Chief	LVC	NCI

Objectives:

To study the evolution of retroviruses within the mammalian genome and to determine the cellular origin of infectious retroviruses. To use the time of interspecies viral transfer as a defined marker for the rates of evolution of various species.

Methods Employed:

Solution hybridization using S₁ nuclease and Southern blot techniques were used to detect double stranded DNA. Primary cell lines from various species were established and immortalized by transformation with sarcoma viruses. Replication of retroviruses was detected by assaying the pellet obtained after high-speed centrifugation of supernatant fluid from cells for reverse transcriptase activity.

Major Findings:1. Carnivore evolution and phylogeny as derived by DNA hybridization.

A phylogenetic tree of the carnivores has been derived from thermal stability measurements of nonrepetitive cellular DNA. The data include 47 species of carnivores (Fissipeds and Pinnepgds); the nonrepetitive cellular DNA of 16 of these species was labelled with ³H-thymidine and hybridized to the other species. Computer programs developed by Drs. Fitch ("Neighborliness") and Dayhoff ("Mattop") were used to derive phylogenies. The data obtained differ in many respects from those obtained by classical methods such as anatomical comparisons and morphometric measurements. For example, the two skunk species examined (Mephitis mephitis and Spilogale putorius), although closely related to each other, are assigned to a separate family within the carnivores. Nucleic acid sequences closely related to those present in the squirrel monkey type D retrovirus genome were shown to be present in skunk DNA but not in the cellular DNA of supposedly closely related species such as mink and weasel. The ancient divergence of skunks from mink, ferret, and weasel ancestors thus explain the data previously obtained with the endogenous squirrel monkey type D retrovirus.

Within the carnivores, the taxonomic relationship of the giant and lesser panda, bears and raccoon has been debated for many years. A phylogenetic tree was derived for these species based on DNA hybridization measurements, genetic distance (based on a comparison of isozyme electrophoretic mobility), immunological distance, and chromosome banding analysis. The data reveal that the lesser panda's ancestors emerged at approximately the time of the raccoon-bear divergence, while ancestors of the giant panda split from the bear lineage much later, just prior to the radiation that led to modern bears.

2. The transfer of retroviruses from primates to felines as a marker for studies of evolutionary rates.

One of the current areas of investigation in molecular biology is the rate at which species accumulate point mutations. Recent reports have revealed that certain species (rodents, for example) accumulate mutations in nonrepetitive DNA at a five- to tenfold faster rate than other mammalian species. One of the difficulties in this type of work is the assignment of precise dates to the emergence of species. We have used the transfer of retroviruses between species, with subsequent incorporation into the germ line, as a marker for evolutionary time that is independent of the fossil record. Earlier work had shown a transfer of retrovirus from primates to cats (with subsequent incorporation into the germ line) sometime during the past several million years. The virus acquired by cats (and six of their descendant species) is called RD-114. We have now shown that this transfer occurred at the time of the gelada-baboon ancestor or about 4 to 6 million years ago. Using this transfer as a marker for evolutionary time, we have studied the rate of accumulation of mutations in those primate and feline species that have diverged since the time of virus transfer. The rate of mutation has been the same in both orders, and the rate of base pair substitutions in DNA is, therefore, independent of generation time in these two mammalian orders. These results allow a direct comparison of recent primate and carnivore divergence times.

Publications:

Benveniste, R. E.: The contributions of retroviruses to the study of mammalian evolution. In MacIntyre, R. J. (Ed.): Molecular Evolutionary Genetics (Monographs in Evolutionary Biology Series). New York, Plenum Press, 1985, pp. 359-417.

O'Brien, S. J., Collier, G. E., Benveniste, R. E., Nash, W. G., Newman, A. K., Simonson, J. M., Eichelberger, M. A., Seal, U. S., Bush, M. and Wildt, D. E.: Setting the molecular clock in Felidae: The great cats, Panthera. In Tilson, R. L. (Ed.): Tigers of the World. Apple Valley, Noyes Publications (In Press)

O'Brien, S. J., Nash, W. G., Wildt, D. E. and Benveniste, R. E.: Riddle of the giant panda phylogeny: A molecular solution. Nature 317: 140-144, 1985.

Patents:

None

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01CP05330-04 LVC

PERIOD COVERED

October 1, 1985 to September 30, 1986

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Urinary Transforming Growth Factors (TGFs) in Human Neoplasia

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Kurt J. Stromberg Medical Director LVC NCI

Others: W. Robert Hudgins Chemist LVC NCI
Raleigh Boaze Bio. Lab. Tech. LVC NCI

COOPERATING UNITS (if any)

Division of Endocrinology, Vanderbilt University, Nashville, TN (D. N. Orth);
Triton Biosciences, Houston, TX (R. Pardue and J. Dedman); Biotope, Inc.,
Bellevue, WA (W. R. Hargreaves)

LAB/BRANCH

Laboratory of Viral Carcinogenesis

SECTION

Viral Leukemia and Lymphoma Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701-1013

TOTAL MAN-YEARS:

2.7

PROFESSIONAL:

1.0

OTHER:

1.7

CHECK APPROPRIATE BOX(ES)

- (a) Human subjects (b) Human tissues (c) Neither
- (a1) Minors
- (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Procedures for concentration and initial separation of TGF- α , EGF, and TGF- β from human urine were developed using adsorption to microparticulate silica (Analytichem, Harbor City, CA, Cat. No. 11021) and fractional elution with increasing concentrations of acetonitrile. Further resolution was obtained by sequential chromatography based on molecular size (Bio-Gel), charge (CM-cellulose), and hydrophobicity (RP-HPLC). The high molecular weight (HMW) of 30,000 to 35,000 Mr, for TGF previously reported in the urine of various cancer patients is, in patients with malignant astrocytomas, indistinguishable from the HMW form of hEGF in terms of apparent molecular size, EGF receptor binding activity, EGF immunoreactivity and clonogenic activity. However, in comparison to bulk (25 liters) urine from normal individuals, equivalently large urine samples from these brain tumor patients contained about fourfold more HMW hTGF/hEGF. hTGF- α was not identified in either source of bulk urine. Secondly, in an *in vitro* study of HMW TGFs, using in part the above resolution scheme, conditioned medium from A673, a human rhabdomyosarcoma cell line, was found to contain principal peaks of EGF radioreceptor and clonogenic activity in SDS-PAGE slices corresponding to Mr 15,000 and 22,000 in an HPLC sample eluting at 25-26% acetonitrile, and two additional higher Mr activities in a 22-23% acetonitrile eluting region. Neither of these active regions from HPLC competed in radioimmunoassay under reduced and denatured conditions for hEGF or rTGF- α when using an antiserum raised against the 17 C-terminal amino acids of rTGF- α . These multiple forms of HMW hTGF- α produced *in vitro* suggest size heterogeneity and possible immunologic diversity among HMW members of the EGF/TGF- α family of growth promoting polypeptides. Thirdly, the urine of patients with disseminated breast cancer contains immunoreactive TGF- α which is not present in comparable control urine from normal individuals. Currently, the pre- and postoperative urine of patients with primary breast cancer without metastasis is being compared for TGF- α activity.

PROJECT DESCRIPTIONNames, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Kurt J. Stromberg	Medical Director	LVC	NCI
W. Robert Hudgins	Chemist	LVC	NCI
Raleigh Boaze	Bio. Lab. Tech.	LVC	NCI

Objectives:

To develop efficient urinary growth factor isolation procedures employing hydrophobic adsorption on microparticulate silica beads, and to isolate and characterize members of a family of epidermal growth factor (EGF)-related urinary growth factors in human cancer patients with respect to their histologic type of cancer and their correlation with the extent of tumor burden.

Methods Employed:

The presence of growth factor(s) was assessed by stimulation of growth of normal rat kidney cells (NRK) in soft agar, competition with ¹²⁵I-labeled mouse EGF (mEGF) for binding to membrane receptors, and radioimmunoassay for human epidermal growth factor (hEGF) and human alpha-TGF (using C17A fragment reagents).

Major Findings:

1. Urinary growth factors adsorb to microparticulate silica. Because urinary transforming growth factors (TGFs) occur as trace peptides in a dilute solution, a sample concentration technique was developed which separates TGF-alpha and TGF-beta from urinary EGF. Characterized growth factors (mouse EGF, human EGF, rat TGF-alpha and human TGF-beta) were iodinated and added to human urine to evaluate the capacity of bonded silica sorbents to separate TGF-alpha and TGF-beta from the high level of background hEGF present in human urine. Commercially available C1 bonded microparticulate silica (Analytichem, Cat. No. 11021) efficiently adsorbed in free suspension ¹²⁵I-labeled mouse EGF, ¹²⁵I-hEGF, ¹²⁵I-rTGF-alpha, and ¹²⁵I-hTGF-beta added to human urine samples. Fractional elution with acetonitrile (MeCN) of the sorbent released approximately 70% to 80% of the ¹²⁵I-mEGF and ¹²⁵I-hEGF between 25% and 30% MeCN, and 80% of the ¹²⁵I-rTGF-alpha between 15% and 25% MeCN, and 70% of the ¹²⁵I-hTGF-beta between 30% and 50% MeCN, with retention after dialysis of less than 0.05%, 0.4%, and 0.3%, respectively, of the original urinary protein. Consequently, a single step enrichment of about 1400-fold for mEGF and hEGF, 200-fold for rTGF-alpha and 250-fold for hTGF-beta was achieved rapidly. Subsequent Bio-Gel P-10 chromatography indicated that ¹²⁵I-mEGF and ¹²⁵I-hEGF eluted later than would be predicted on the basis of their reported molecular weights of approximately 6,000, while ¹²⁵I-hTGF-beta eluted from Bio-Gel P-10 at an approximate Mr 24,000, and ¹²⁵I-rTGF-alpha eluted at an approximate Mr of 8,000 to 9,000. ¹²⁵I-rTGF-alpha and ¹²⁵I-hTGF-beta bound to carboxymethyl-cellulose and eluted at a different pH and ionic strength than hEGF. On reverse phase HPLC, using a linear gradient of 18% to 35% MeCN over 120 min., ¹²⁵I-rTGF-alpha was comparatively hydrophilic (eluting at 22% MeCN) in contrast to ¹²⁵I-hEGF (eluting at 27% MeCN) and the very hydrophobic hTGF-beta (which eluted at 34%

MeCN). These observed biochemical differences among TGF- α , EGF, and TGF- β provide a rationale for resolution of these and other EGF-related growth factors from bulk urine of normal individuals and tumor-bearing patients.

2. Human brain tumor-associated urinary high molecular weight (HMW) growth factor of 33,000 Mr is identical to human HMW epidermal growth factor. Urine was obtained from a patient who had a highly malignant brain tumor (astrocytoma, grade IV). Following adsorption to microparticulate silica beads and elution with acetonitrile to yield an HMW human transforming growth factor (hTGF), this HMW hTGF promoted clonogenic cell growth in soft agar and competed for membrane receptors with mouse EGF. The growth factor activity eluted from beads at 25-27% acetonitrile, and after purification by Bio-Gel P-100 chromatography and high performance liquid chromatography (HPLC), had a molecular weight of approximately 33,000 on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), identical to that of a highly purified HMW form of human EGF (HMW hEGF) previously reported to be present in trace concentrations in normal human urine. Following surgical resection of the tumor, no appreciable HMW hTGF activity was detectable in urine. HMW hTGF generated a competitive binding curve similar to that of hEGF and parallel to that of HMW hEGF. Both hEGF and HMW hEGF were clonogenic in soft agar, and their clonogenic activity, as well as that of HMW hTGF, was inhibited by hEGF antiserum. Both HMW hTGF and HMW hEGF had 20 to 25% of the radioreceptor binding activity of hEGF. Thus, HMW hTGF was indistinguishable from HMW hEGF in terms of apparent molecular size, EGF receptor binding activity, EGF immunoreactivity and clonogenic activity.

3. Characterization of EGF-related HMW transforming growth factors secreted by A673 cells. Extracts of serum-free conditioned medium from A673, a human rhabdomyosarcoma cell line, contain HMW transforming growth factors (HMW TGFs) after partial purification by Bio-Gel P-100 and carboxymethyl cellulose chromatography (Todaro, G. J., Fryling, C. M. and De Larco, J. E.: Proc. Natl. Acad. Sci. USA 77: 5258, 1980). Further characterization with reverse-phase HPLC revealed a principal peak of coincident EGF receptor competition and soft agar activity at 25-26% acetonitrile. If a side shoulder of EGF radioreceptor activity from the carboxymethyl cellulose chromatography was also included for subsequent HPLC analysis, additional active fractions were observed at 22-23% acetonitrile. Importantly, both active regions from HPLC failed to compete in radioimmunoassays under reduced and denatured conditions for hEGF or rat TGF- α , and failed to give positive signals in Western blots under conditions in which hTGF- α was readily detected when using an antisera raised against the 17 C-terminal amino acid of rTGF- α . Nonreducing SDS-PAGE of the HMW TGFs from HPLC revealed EGF radioreceptor and soft agar activity in gel slices corresponding to M_r 15,000 and 22,000 in the principal TGF activity from HPLC eluting at 25-26% acetonitrile and M_r 15,000, 20,000, 27,000 and 50,000 in the active HPLC fractions eluting at 22-23% acetonitrile.

4. Urine from patients with disseminated breast cancer contains authentic, immunoreactive TGF- α . A 22-liter pool of urine from patients with widely disseminated adenocarcinoma of the breast was processed as above to yield a TGF- α -like activity which eluted from carboxymethyl cellulose chromatography and reverse phase HPLC in a manner identical to ^{125}I -rTGF- α . Importantly, this purified hTGF- α gave a competition curve parallel to synthetic ^{125}I -rTGF- α in radioimmunoassay. No comparable activity was observed in a 30-liter pool of urine from normal control individuals.

Publications:

- Benveniste, R. E., Stromberg, K., Morton, W. R., Tsai, C. C. and Giddens, W. E., Jr.: Association of retroperitoneal fibromatosis with type D retroviruses. In Salzman, L. (Ed.): Animal Models of Retrovirus Infection. New York, Academic Press, 1986, pp. 335-354.
- Hudgins, W. R. and Stromberg, K.: Radioisotopic quantitation of efficient separation of urinary peptide growth factors using C1 bonded silica. In Lewis, S. (Ed.): Proceedings of the Fourth Annual Symposium on Sample Preparation and Isolation Using Bonded Silicas. Harbor City, California, Analytichem International (In Press)
- Ruscetti, F. W., Kalyanaraman, V. S., Stevenson, H., Stromberg, K., Herberman, R. B., Farrar, W. and Ortaldo, J. D.: Natural killer cells and other leucocytes as effector cells against HTLV-I and LAV/HTLV-III infected lymphoid cells. J. Immunol. 36: 3619-3624, 1986.
- Stromberg, K., Benveniste, R. E., Arthur, L. O., Rabin, H., Giddens, W. E., Jr., Ochs, H. D., Morton, W. R. and Tsai, C. C.: Characterization of exogenous type D retrovirus from a fibroma of a macaque with simian AIDS and fibromatosis. In Kulstad, R. (Ed.): Papers from Science. Washington, D.C., American Association for the Advancement of Science, 1986, pp. 136-142.
- Stromberg, K. and Hudgins, W. R.: Urinary transforming growth factors in neoplasia: Separation of ^{125}I -TGF-alpha from EGF in human urine. Cancer Res. (In Press)
- Stromberg, K., Hudgins, W. R., Dorman, L. S., Henderson, L. E., Sowder, R. C., Orth, D. N., Sherrell, B. J. and Mount, C. D.: Urinary transforming growth factors (TGFs) in human neoplasia: Human brain tumor-associated urinary high molecular weight (HMW) TGF is identical to HMW epidermal growth factor. Cancer Res. (In Press)
- Stromberg, K., Hudgins, W. R., Fryling, C. M., Dedman, J. R., Pardue, R. L., Hargreaves, W. R. and Orth, D. N.: Characterization of alpha type transforming growth factors secreted by human A673 cells. J. Cell. Biochem. (In Press)

Patents:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05333-04 LVC

PERIOD COVERED

October 1, 1985 to September 30, 1986

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Evolutionary Relationships of the Felidae: A Mitochondrial DNA Approach

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	Stephen J. O'Brien	Chief	LVC	NCI
Others:	David E. Wildt	Guest Researcher	LVC	NCI
	Lisa Forman	Guest Researcher	LVC	NCI

COOPERATING UNITS (if any)

Department of Biochemistry, Howard University, Washington, DC (M. George, Jr., E. T. Butler)

LAB/BRANCH

Laboratory of Viral Carcinogenesis

SECTION

Genetics Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701-1013

TOTAL MAN-YEARS:

1.4

PROFESSIONAL:

1.2

OTHER:

0.2

CHECK APPROPRIATE BOX(ES)

- (a) Human subjects (b) Human tissues (c) Neither
 (a1) Minors
 (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

This research seeks to clarify the relationship between the chromosomal, protein and DNA topologies among the Felidae. Specifically, we are investigating the entire cat family using mitochondrial DNA (mtDNA) as an evolutionary probe in order to more finely resolve previous felid phylogenies. mtDNA has proven to be an excellent metric for elucidating evolutionary relationships because it is a rapidly evolving molecule that is entirely maternally inherited (i.e., the data are not confounded by meiotic events) and it affords a high degree of differentiation between closely related species. mtDNA has been extracted from several individuals in each of five target species: domestic cat, cheetah, clouded leopard, lion and Geoffrey's cat. mtDNA cleavage maps have been constructed for all target species using 17 restriction enzymes for an average of 43 sites per map. Intra- and interspecific variation has been calculated from these maps. The mtDNA of the domestic cat has been molecularly cloned into the pBR322 plasmid for use as a nick-translated probe in the "Southern" analysis of high molecular weight DNA from 35 of the 37 cat species. The resulting restriction fragment length patterns from this analysis are being used to construct a mtDNA phylogeny of the Felidae in toto. This dendrogram of evolutionary relationships will be compared to other topologies of the Felidae derived by other molecular and morphometric metrics.

PROJECT DESCRIPTIONNames, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Stephen J. O'Brien	Chief	LVC	NCI
David E. Wildt	Guest Researcher	LVC	NCI
Lisa Forman	Guest Researcher	LVC	NCI

Objectives:

The determination of the phylogenetic relationships of the Felidae using mitochondrial DNA (mtDNA) as an evolutionary probe. An estimate of the amount and type of inter- and intraspecific variation in mtDNA among the Felidae. The characterization of certain target species by detailed mtDNA cleavage maps. The molecular cloning of the mtDNA genome of the domestic cat to probe for restriction fragment length polymorphisms in *Felis catus* and to use as a molecular probe for characterizing the mtDNA found in the high molecular weight DNA of 35 other felid species representing virtually the entire Felidae.

Methods Employed:

Mitochondrial DNA (mtDNA) was isolated and purified from tissue organs by differential centrifugation. This procedure also involves the use of CsCl density gradients. The purified mtDNA was subjected to digestion with 17 different restriction enzymes. The mtDNA fragments were end-labeled with ³²P using DNA polymerase I (large fragment) and separated by electrophoresis on vertical agarose slab gels. After autoradiography of the vacuum-dried gels, the base-pair lengths of the fragments and genome sizes were determined. Construction of the cleavage maps involved multiple enzyme digestions. Other methods included the isolation and preparation of high molecular weight DNA from felid cultured cell lines, preparation and purification of mtDNA fragments for molecular cloning, nick-translation of mtDNA clones, and "Southern" blot analysis.

Major Findings:

mtDNA cleavage maps from five target species (domestic cat, cheetah, clouded leopard, lion and Geoffrey's cat) have been constructed using 17 restriction enzymes. These 17 enzymes yielded an average of 43 sites per map and reveal that felid mtDNA is slightly larger than that of human, mouse or cow. However, overall the cat mtDNA genome shows a great deal of conservation with other vertebrates in its gene order and restriction enzyme sites.

A comparison of the cleavage maps from the five target species revealed a high degree of intergenetic variation:

<u>Species Compared</u>	<u>% Sequence Variation</u>
Domestic cat vs. cheetah	11%
Domestic cat vs. lion	25%
Domestic cat vs. clouded leopard	25%

Domestic cat vs. Geoffrey's cat	25%
Cheetah vs. lion	25%
Cheetah vs. clouded leopard	25%
Cheetah vs. Geoffrey's cat	25%
Lion vs. clouded leopard	15%
Geoffrey's cat vs. lion	13%
Geoffrey's cat vs. clouded leopard	15%

This degree of differentiation is much higher than that reported by the DNA:DNA hybridization technique.

Intraspecific variation has been examined in 25 domestic cats by end-labelling and Southern blotting methods. These analyses identified only a small amount of intraspecific variation, although three distinct mtDNA morphs could be consistently identified. Intraspecific variation in exotic cats (three cheetahs, two clouded leopards) by end-labeling methods showed no variation between individuals. However, these results must be considered preliminary until more individuals can be examined by Southern blotting procedures.

Domestic cat mtDNA has been cloned into a plasmid vector (pBR322) using BamHI and EcoRI sites to generate three fragments. Pilot studies assessing the sensitivity of our nick-translated probes to the mtDNA found in cellular DNAs isolated from domestic and exotic cat species have been completed. These studies affirm the utility of this technique when minor modifications are made in DNA extractions from crude tissue and cell culture. An in-depth study of the Panthera lineage (lion, tiger, leopard, snow leopard, jaguar, and cougar) is currently being conducted to develop estimates of interspecific variation and clarify the systematics of this group.

Publications:

George, M., Jr. and Ryder, O. A.: Mitochondrial DNA evolution in the genus Equus. Mol. Biol. Evol. (In Press)

Higuchi, R., Wrischnik, L., Oakes, E., George, M., Tong, B. and Wilson, A. C.: Phylogenetic position of the extinct quagga: Evidence from mitochondrial DNA. Nature (In Press)

Patents:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01CP05367-02 LVC	
PERIOD COVERED October 1, 1985 to September 30, 1986			
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) The Genetic Structure of Natural Populations of Past and Present			
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)			
PI:	Stephen J. O'Brien	Chief	LVC NCI
Others:	Janice S. Martenson	Microbiologist	LVC NCI
	Mary A. Eichelberger	Microbiologist	LVC NCI
	William S. Modi	Staff Fellow	LVC NCI
	Lisa Forman	Guest Researcher	LVC NCI
COOPERATING UNITS (if any) Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil (H. Seuanez); Laboratory of Clinical Studies, ALC, NIH, Bethesda, MD (D. Goldman); National Zoological Park, Washington, DC (M. Bush, D. E. Wildt); National Museums of Kenya, Nairobi, Kenya (R. E. Leakey); PRI, Frederick, MD (R. K. Wayne)			
LAB/BRANCH Laboratory of Viral Carcinogenesis			
SECTION Genetics Section			
INSTITUTE AND LOCATION NCI, NIH, Frederick, Maryland 21701-1013			
TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:	
2.3	1.8	0.5	
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input checked="" type="checkbox"/> (b) Human tissues <input type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews			
SUMMARY OF WORK (Use standard unredacted type. Do not exceed the space provided.) <p>Population genetic analysis of human and animal populations have been used to study the genetic health and disease susceptibility of several species. Over 30 polymorphic human loci detected by 2DE have been described. The South African subspecies of cheetah was shown to be genetically depauperate insofar as it is monomorphic at 52 isozyme loci. Unrelated cheetahs also accepted skin grafts, a situation without precedent among outbred mammalian species. A devastating epizootic of the species of feline infectious peritonitis (FIP and RNA containing corona virus) was hypothesized to result from abrogation of a major histocompatibility complex (MHC) haplotype in T-cell stimulation. A sample of the east African cheetah subspecies showed similar genetic status and prompted us to hypothesize the occurrence of two severe population contractions in the natural history of the species. A molecular phylogeny of the great and lesser apes and man was derived based on genetic distance of 383 different proteins resolved by 2DE. A molecular phylogeny of the 37 species of the Felidae was constructed based on immunological distance using serum albumin. Similarly, a consensus phylogeny of the Ursidae <u>Ailuropoda</u> (giant panda) and <u>Ailurus</u> (lesser panda) was derived from distance matrices derived from three distinct molecular measures of genetic distance. A reconstruction experiment of organismal evolution using inbred mouse strain of defined genealogy demonstrated that morphologic and molecular genetic variation are uncoupled. A comparative analysis of cytological and linkage maps of mammals have indicated a noncontinuous tempo of chromosomal evolution in certain lineages (e.g., primates, felids) that are highly conserved in their chromosomal presentation, while others (rodents, lesser apes, canids) are chromosomally shuffled as if rapid saltatory cytological rearrangements occurred during the speciation events.</p>			

PROJECT DESCRIPTIONNames, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Stephen J. O'Brien	Chief	LVC	NCI
Janice S. Martenson	Microbiologist	LVC	NCI
Mary A. Eichelberger	Microbiologist	LVC	NCI
William S. Modi	Staff Fellow	LVC	NCI
Lisa Forman	Guest Researcher	LVC	NCI

Objectives:

1. Development of molecular procedures for assessing genetic status of natural populations and for use in studying heritability of disease susceptibility, both congenital and etiologic.
2. Use of molecular procedures to determine phylogenetic affinities and relationships between extant species of hominoid primates, felids and selected carnivores. The derived topologies have important implications for heterologous embryo transfer and more generally for the ultimate resolution of interacting gene systems that drive development and cancer.
3. The biologic resolution of adaptive strategies employed by rarely studied mammalian populations for defense against neoplastic etiologic agents that affect human populations.

Methods Employed:

The following techniques were employed: (1) cell culture procedures, (2) isozyme electrophoresis, (3) two-dimensional gel electrophoresis, (4) microcomplement fixation using heterologous rabbit antisera, (5) high resolution cytogenetics procedures, (6) gene mapping procedures using somatic cell hybrids, (7) virological procedures, (8) surgical skin grafting, and (9) statistical analysis of phylogenetic algorithms.

Major Findings:

1. Identification of a family of polymorphic human loci which encode soluble proteins resolved by two-dimensional gel electrophoresis. Twenty-seven independent polymorphic loci were detected by two-dimensional electrophoresis (2DE) of serum, erythrocytes, and fibroblasts in two large families and analyzed for linkage to classical genetic markers. We detected 7 serum, 4 erythrocyte and 17 fibroblast protein loci that exhibited charge variation in these two families and in a sample of unrelated individuals. The genetic basis of protein variants was confirmed by quantitative gene dosage dependence and by conformance to Mendelian transmission in the two families, except for four rare variants for which transmission analysis was not possible. Linkage analysis demonstrated that each of the variants represent products of independent loci, with the exception of erythrocyte locus (RBC4), which we also detected in fibroblasts (NC27). Two allozyme polymorphisms, glyoxalase-1 (GL01) and phosphoglucomutase-3 (PGM3), were

specifically identified here based on genotypic concordance and molecular mass. Unknown fibroblast protein (NC22) may be linked to apolipoprotein E (Lod score = 2.8 at $0^m=.1, 0^f=0$). These studies indicate a minimum level of average protein charge heterozygosity of approximately 2.2% for the most predominant human cellular proteins and of 5.6% for the most predominant proteins of serum.

2. Derivation of a molecular phylogeny of Primates, Felidae and Ursidae. A molecular phylogeny for the hominoid primates was constructed using protein genetic distances from a survey of 383 radiolabeled fibroblast polypeptides resolved by 2DE. An internally consistent matrix of Nei-genetic distances was generated on the basis of variants in electrophoretic position. The derived phylogenetic tree indicated a branching sequence, from oldest to most recent, of cercopithecoids (Macaca mullata), gibbon-siamang, orangutan, gorilla and human-chimpanzee. Hominid molecular distance measures obtained by 2DE are fairly consistent with those generated with other molecular procedures except that the gorilla and orangutan lineages were found to have diverged from human-chimpanzee at nearly the same time [12 to 16 million years (my) before the present (BP)].

The phylogenetic distances between 34 of the 37 extant species of Felidae were estimated using albumin immunological distances (AID). Albumins from ten cat species were used to prepare antisera in rabbits. A consensus phylogeny was constructed from a matrix of reciprocal AID measurements using four distinct phylogenetic algorithms. The derivative topology demonstrated that the cat family evolved as three major lineages. The first (around 13 my BP) led to the small South American cats (ocelot, margay, etc.), the second led to the small Mediterranean cats related to the domestic cat (8 to 10 my BP) and the last led to the great cats. An independently derived topology derived from genetic distance using 50 isozyme loci confirmed these conclusions.

The taxonomic status of the giant panda and the lesser panda have been a biological puzzle since their description by western naturalists a century ago. Although there has been some agreement that the lesser panda was a member of the raccoon family (Procyonidae), the giant panda has been classified with almost equal frequency in Ursidae (bear family), in Procyonidae, and as a single member of a separate family, Ailuropodidae. The results of four independent molecular genetic approaches, DNA hybridization, isozyme genetic distance, immunological distance and G-based karyology, were performed and converged on a consensus phylogeny of the pandas based upon the principles of the "molecular clock" hypothesis. The lesser panda diverged from New World procyonids just subsequent to their departure from ursids, while ancestors of the giant panda's split from the ursid lineage just prior to the radiation that led to modern bears. The giant panda's divergence was accomplished by a chromosomal reorganization that can be partially reconstructed from the ursid karyotype, but not from procyonids or the lesser panda. The apparently dramatic, but actually limited distinctions between the giant panda and the bears in chromosomal and anatomical morphology provide a graphic mammalian example of the discordance of molecular versus morphological (and chromosomal) evolutionary change.

3. Empirical demonstration that evolution of molecular and morphological variation in mammals are uncoupled. The relationship between morphometric and structural gene variation was examined in a group of 15 inbred mouse strains. The genetic distance of Nei was calculated between each of the strains based upon 36

to 78 biochemical loci. Mahalanobis distances based on 11 mandibular measurements were computed between the strains as well. The correlation coefficient between genetic distance and metric distance was low ($r = 0.12 \pm 0.1$). Genetic distance was highly correlated with elapsed time of divergence of the strains ($r = 0.73 \pm 0.15$) while morphometric distance, though clearly heritable, was not significantly correlated with elapsed time. These results indicate that morphological change and biochemical change are poorly coupled in these strains and suggest that the cumulative differences in structural gene mutations may provide a more accurate measure of phylogenetic relationships between biological groups than do measures of morphological distance (such as mandibular characters) based upon multivariate analysis.

4. Genetic demonstration that the African cheetah suffered two extreme population bottlenecks in its natural history, an ancient Pleistocene contraction and a recent (within 200 years) local founder effect. We had recently discovered that the southern African cheetah (*A. jubatus jubatus*) is rather unusual among felids and other mammals in having an extreme paucity of genetic variation as estimated by electrophoretic surveys of allozymes and cell proteins resolved by two-dimensional gels. Possibly even more unusual was the observation that 14 cheetahs accepted allogeneic skin grafts from unrelated cheetahs revealing genetic monomorphism at the major histocompatibility complex, an abundantly polymorphic locus in nearly all mammals. These genetic observations prompted us to hypothesize that the cheetah species had likely suffered a demographic contraction or population bottleneck necessarily followed by inbreeding in its recent history. All these conclusions had been based on our samples of animals from the south African subspecies, *Acinonyx jubatus jubatus*. This year, a combined population genetic and reproductive analysis of free-ranging cheetahs from East Africa (*A. jubatus raineyi*) was undertaken to compare them with the genetically impoverished and reproductively impaired southern Africa subspecies (*A. jubatus jubatus*). The quality of semen specimens from east African cheetahs was poor with low concentration of spermatozoa ($20 \times 10^6/\text{ml}$) and high incidence of morphological abnormality (79%). Two allozyme polymorphisms in *A. j. raineyi* were detected and one of these shows a rare allele (less than 1%) in south African specimens. The genetic distance (D) between southern and east African cheetahs was low ($D = 0.004$), suggesting that the development of relative genetic uniformity preceded the recent (within 100 years) geographic isolation of the subspecies. We propose that at least two population bottlenecks followed by inbreeding of cheetahs produced the modern species; the first and most extreme was ancient, possibly late Pleistocene (circa 10,000 years ago), the second more recent (within the last century) and leading to the southern African populations.

5. Resolution of the tempo and mode of genomic and chromosomal evolution in mammals. The cumulative analysis of the synteny gene maps of over 20 mammalian species (see Genetic Maps, 1984) and the comparative cytological appearance of over 100 species has led to the following consensus. Mammalian orders vary tremendously with respect to conservation of chromosome presentation. Certain mammalian families show extreme conservation of linkage and chromosomal integrity. For example, within the cat family, Felidae, 90% of the chromosomes are cytologically identical between each of the 37 member species. A second Carnivore family, the dogs (Canidae) is characterized by extremely variable chromosomal numbers and appearance. With certain dramatic exceptions (e.g., gibbon and owl monkey), the primates have conserved chromosomal morphologies that have allowed

the tracing of chromosome phylogenies as far back as the prosimians. The rodents, like the dogs, gibbons, bears and others, have a highly shuffled or rearranged karyotype within the order, within genera and even within the same species. After reviewing these observations, an inevitable conclusion is that mammalian chromosomal evolution is not continuous; rather, it is punctuated with massive complex rearrangements in certain phyla but with highly conserved arrangements in others. An attempted reconstruction of the cytological rearrangements that have taken place during evolution is in progress and will be compared to nonspecific chromosomal aberrations that are seen in human cancers.

6. The severely threatened golden lion tamarin (*Leontopithecus rosalia rosalia*) is genetically impoverished. The golden lion tamarin (*Leontopithecus rosalia rosalia*, one of the rarest and most endangered of New World primates, has been the focus of an intensive research and conservation effort for two decades. During that period managed breeding from 44 founders has brought the captive population to over 400 individuals, a number that equals or exceeds the estimated number of free-ranging golden lion tamarins. The extent of genetic variation among captive and free-ranging golden lion tamarins was estimated by electrophoretic survey of 47 allozyme loci from 83 individuals. The amount of variation was low when compared to 15 other primate species with four percent of the loci being polymorphic (P), and with an average heterozygosity (H) estimate of 0.1. Analysis of captive animals of two allopatric morphotypes, *Leontopithecus rosalia chrysopygus* and *Leontopithecus rosalia chrysomelas* were similar to the *rosalia* findings insofar as they also retained limited genetic polymorphism. Computation of the Nei-genetic distance measurements revealed that the three morphotypes were genetically very similar. These data are consistent with the occurrence of reproductive isolations within recent times possibly contemporaneous with and caused by the predominance, of human culture in South America.

Publications:

Forman, L., Kleiman, D., Bush, M., Ballou, J., Phillips, L., Dietz, J. L., Coimbra-Filho, A. and O'Brien, S. J.: Genetic variation within and among lion tamarins. Am. J. Phys. Anthropol. (In Press)

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Modi, W. S.: Chromosomes of six species of New World microtine rodents. Mammalia 49: 357-363, 1985.

Modi, W. S.: Karyotypic differentiation among two sibling species pairs of New World microtine rodents. J. Mammal. 67: 159-166, 1986.

Modi, W. S.: Reproductive tactics among deer mice of the genus *Peromyscus*. Can. J. Zool. 62: 2576-2581, 1985

Newman, A., Bush, M., Wildt, D. E., van Dam, Dirk, Frankehuis, M., Simmons, L., Phillips, L. and O'Brien, S. J.: Biochemical genetic variation in eight endangered feline species. J. Mammal. 66: 256-267, 1985.

O'Brien, S. J., Benveniste, R. E., Nash, W. G., Simonson, J. M., Eichelberger, M. A., Wildt, D. E. and Bush, M.: Constructing a molecular phylogeny of the giant panda, Ailuropoda melanoleuca. In Klos, H.-G. and Fradrich, H. (Eds.): Proceedings International Symposium on Giant Panda, 1984. Berlin, Bongo, 1985, Vol. 10, pp. 175-182.

O'Brien, S. J., Collier, G. E., Benveniste, R. E., Nash, W. G., Newman, A. K., Simonson, J. M., Eichelberger, M. A., Seal, U. S., Bush, M. and Wildt, D. E.: Setting the molecular clock in Felidae: The great cats, Panthera. In Tilson, R. L. (Ed.): Tigers of the World. Apple Valley, Noyes Publications (In Press)

O'Brien, S. J., Nash, W. G., Wildt, D. E., Bush, M. E. and Benveniste, R. E.: A molecular solution to the riddle of the giant panda's phylogeny. Nature 317: 140-144, 1985.

O'Brien, S. J., Wildt, D. E. and Bush, M.: The African cheetah in genetic peril. Sci. Am. (May): 84-92, 1986.

Schmidly, D. J., Lee, M. R., Modi, W. S. and Zimmerman, E. G.: Systematics and notes on the biology of Peromyscus hooperi. Occ. Pap. Mus. Texas Tech. Univ. 97: 1-40, 1985.

Wayne, R. K.: Cranial morphology of domestic and wild canids: The influence of development on morphological change. Evolution 40: 243-261, 1986.

Wayne, R. K., Forman, L., Newman, A. K., Martenson, J. S. and O'Brien, S. J.: Genetic monitors of captive zoological populations: Morphological and electrophoretic assays. Zoo Biol. (In Press)

Wayne, R. K. and O'Brien, S. J.: Empirical demonstration that structural gene and morphometric variation between mouse strains are uncoupled. J. Mammal. (In Press)

Wayne, R. K. and O'Brien, S. J.: The extent and character of morphologic variation in the cheetah (Acinonyx jubatus), a genetically uniform species. Evolution 40: 78-85, 1986.

Wildt, D. E., Schiewe, M. C., Schmidt, P. M., Goodrowe, K. L., Howard, J. G., Phillips, L. G., O'Brien, S. J. and Bush, M.: Developing animal model systems for embryo technologies in rare and endangered wildlife. Theriogenology 25: 33-51, 1986.

Patents:

None

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01CP05382-03 LVC

PERIOD COVERED

October 1, 1985 to September 30, 1986

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Genes Involved in Preneoplastic Progression

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	Nancy H. Colburn	Chief, Cell Biology Section	LVC	NCI
Others:	W. Karol Dowjat	Visiting Fellow	LVC	NCI
	Cao Ya	Guest Researcher	LVC	NCI
	Michael Antecol	Visiting Fellow	LVC	NCI
	Glenn Hegamyer	Health Science Officer	LVC	NCI

COOPERATING UNITS (if any) Hunan Med. College, Hunan, China (K.-T. Yao); Cancer Res. Lab., Univ. W. Ontario, Canada (D. Denhardt); Dept. Radiation Oncology, Univ. Arizona Med. Sch., Tuscon, AR (T. Bowden); German Cancer Res. Ctr., Heidelberg, W. Ger. (P. Kreig); PRI, Frederick, MD (R. Garrity); NIDR, NIH, Bethesda, MD (M. Lerman)

LAB/BRANCH

Laboratory of Viral Carcinogenesis

SECTION

Cell Biology Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701-1013

TOTAL MAN-YEARS:

4.9

PROFESSIONAL:

2.5

OTHER:

2.4

CHECK APPROPRIATE BOX(ES)

- (a) Human subjects (b) Human tissues (c) Neither
- (a1) Minors
- (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The aim of this research is to identify and characterize genes that specify susceptibility to tumor promoter-induced neoplastic transformation in mice and humans. Evidence suggesting the involvement of such genes in animal and human systems has come from the observation that animals can be bred for sensitivity to tumor promotion. Two genes that specify sensitivity to promotion of neoplastic transformation by tumor promoters in mouse epidermal JB6 cells have been previously cloned. These putative genes, termed pro-1 and pro-2, have been sequenced and are being characterized with respect to mode of activation and regulation of expression. Genomic DNA of a Chinese nasopharyngeal carcinoma cell line, CNE2, confers promotion sensitivity (P+) activity on resistant mouse cells. This activity is, at least in part, attributable to activated homologs of mouse pro-1, as shown by screening a CNE2 genomic library with a mouse pro-1 probe and testing the homologs for P+ activity after transfection into resistant mouse cells. Inactive pro-1 homologs isolated from a normal human library and from the CNE2 library are being compared with activated CNE2 pro-1 to ascertain the mode of activation. Two cDNA libraries, one from initiation-promotion induced skin papillomas and the other from a squamous carcinoma, have yielded clones homologous to pro-2, each containing a 2.1 kb cDNA. This finding is significant in that it not only facilitates intron-exon assignment in the genomic clone but also opens up investigation of the role of pro-2 expression in carcinogenesis in vivo.

PROJECT DESCRIPTIONNames, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Nancy H. Colburn	Chief, Cell Biology Section	LVC	NCI
W. Karol Dowjat	Visiting Fellow	LVC	NCI
Cao Ya	Guest Researcher	LVC	NCI
Michael Antecol	Visiting Fellow	LVC	NCI
Glenn Hegamyer	Health Science Officer	LVC	NCI

Objectives:

To elucidate the nature of genetically determined events that are causally related to preneoplastic progression in mice and humans. To clone the genes involved in tumor promoter-induced progression to the tumor cell phenotype in JB6 mouse epidermal cells. To elucidate the structure and mode of activation of these genes. To learn how expression of these genes is regulated. To learn the function(s) of these genes and their products. To clone and characterize activated human homologs of promotion sensitivity genes. To characterize genes that specify "cancer proneness" in certain genetically cancer-prone conditions. To clone and characterize a novel transforming gene and its regulation by pro genes.

Methods Employed:

The following techniques are being utilized: (1) gene cloning techniques using sib selection search routines; (2) calcium-phosphate DNA transfection followed by assay of sensitivity to promotion of anchorage-independence by the tumor promoter, TPA; (3) use of ligated dominant selectable markers; (4) generation of new recombinant DNA constructs; (5) restriction mapping; (6) sequencing the cloned genes by the Maxam and Gilbert technique; (7) computer-aided analysis of their structure and possible function; (8) Southern and Northern transfer techniques to analyze genome organization and expression of the pro genes; (9) purification of nuclear and messenger RNA; (10) construction of genomic and cDNA libraries; and (11) screening of libraries for pro gene homologs.

Major Findings:

The discovery of two putative genes that specify susceptibility to tumor promoter-induced neoplastic transformation and which may function to trigger the constitutive expression of a novel transforming gene is being extended.

1. Sequencing of mouse genomic pro-2 and isolation of a pro-2 cDNA clone from a mouse carcinoma library. Sequencing of mouse promotion sensitivity gene pro-2 has revealed that it is 3,699 nucleotide pairs long and is unique except for a 536 base pair repeated sequence (designated HLS) bounded by EcoRI. This HLS repeat hybridizes to 0.1 and 1 percent of the clones, respectively, in each of two mouse fibroblast libraries and about 0.004% of the clones in each of three human libraries. The HLS repeat is unrelated to known mouse repeats and apparently represents a new class of mouse repeated sequences. A mouse epidermal squamous

cell carcinoma cDNA library and a papilloma cDNA library prepared from tumors induced by an initiation-promotion protocol by G. T. Bowden, Univ. of Arizona, and P. Kreig, German Cancer Research Center, Heidelberg, have been screened for cDNA clones hybridizing to a mouse pro-2 probe. The probe used contained the entire genomic clone pro-2 sequence minus the 536 bp HLS sequence. This hybridizes to unique restriction fragments in a Southern analysis of mouse genomic DNA. The screening revealed pro-2 hybridizing cDNA clones in both the papilloma and the carcinoma libraries. Four randomly chosen plaque-purified clones show pro-2 hybridizing cDNA inserts of 2.1 kb. This finding is significant in that it not only facilitates intron-exon assignment in the genomic clone but also opens up investigation of the role of pro-2 expression in carcinogenesis in vivo.

2. Characterization of activated human homologs of pro-1. All of five normal mouse DNAs and three normal human DNAs were inactive in transferring promotion sensitivity to JB6 P⁻ cells. Several human sarcoma or carcinoma DNAs also lacked P⁺ activity. In contrast, the genomic DNA of both of two Chinese nasopharyngeal carcinoma cell lines, CNE₁ and CNE₂, when transferred by calcium phosphate transfection, conferred promotion sensitivity on JB6 P⁻ cells. The molar-specific activity of these DNAs was similar to that of mouse P⁺ cellular DNA. To address the question of whether the P⁺ activity associated with nasopharyngeal carcinoma cell DNA could be attributed to homologs of mouse pro-1 or pro-2, a genomic library of CNE₂ DNA was constructed and screened. Screening the CNE₂ library with mouse probes indicated the presence of up to 20 pro-1 homologs per genome and close to one pro-2 homolog per genome. All three of three CNE₂ pro-2 clones tested lacked P⁺ activity on transfection into JB6 P⁻ cells, while all but one of the CNE₂ pro-1 clones tested were activated. Thus, the P⁺ activity associated with CNE₂ DNA can, at least in part, be attributed to homologs of pro-1. This suggests a possible role for activated pro-1 in the etiology of nasopharyngeal carcinoma. Progress is being made toward understanding the mode of activation of human pro-1. Activation by gross rearrangements has been ruled out. The minimum sequence specifying P⁺ activity is 3.4 kb or shorter. This is being compared by chimeric plasmid and sequence analysis with a closely related but inactive fragment from a nonactivated CNE₂ pro-1 clone, as well as with a pro-1 homolog from a normal human sperm library.

3. pro-1, in addition to transferring promotion sensitivity, can transfer activity for life span extension. Fibroblasts of basal cell nevus syndrome (BCNS) patients, but not age-matched normal fibroblasts, can be induced to escape senescence by transfection of pro-1 or v-myc DNA. After transfection of p26 (pro-1) or v-myc plasmid DNAs, BCNS fibroblasts, but not age, race and sex-matched normal fibroblasts, have undergone at least 20 more population doublings than untransfected controls that experienced senescence. Evidence for integration of the pro-1 plasmid into BCNS cellular DNA after transfection has been obtained using probes to the vector. This suggests that pro-1 or v-myc genes, in cooperation with BCNS gene(s), may be able to function in extending longevity of human fibroblasts in culture, which may lead to the establishment of a cell strain, an event postulated to be a component of neoplastic transformation. Thus, pro-1 may cooperate with BCNS gene(s) to produce life span extension and with genes activated in JB6 P⁻ cells to produce neoplastic transformation.

4. A novel transforming activity associated with nasopharyngeal carcinoma DNA. The DNA of CNE₁ and CNE₂ cells, when transfected into JB6 P⁺ CL 41 cells, confers anchorage-independent transformation. This transforming activity resembles that associated with the DNA of tumorigenic TPA-transformants of mouse P⁺ cells designated RT101 and T³⁶²⁷⁴. Both the RT101 (and the T³⁶²⁷⁴) cell DNA and the human CNE DNA transform JB6 P⁺ but not NIH 3T3 cells. Both DNAs also confer promotion sensitivity on JB6 P⁻ cells. This raises the possibility that the human CNE cells may be analogs of tumorigenic JB6 transformants and may offer a human system for studying a class of transforming genes regulated by promotion sensitivity genes.

Publications:

Colburn, N. H.: Genes and membrane signals involved in neoplastic transformation. In Huberman, E. (Ed.): Carcinogenesis. New York, Raven Press, 1985, Vol. 10, pp. 235-248.

Colburn, N. H.: The genetics of tumor promotion. In Barrett, J. C. (Ed.): Mechanisms of Environmental Carcinogenesis. CRC Press, Inc. (In Press)

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Lerman, M. I., Hegamyer, G. A. and Colburn, N. H.: Cloning and characterization of putative genes that specify sensitivity to induction of neoplastic transformation by tumor promoters. Int. J. Cancer 37: 293-301, 1986.

Shimada, T., Gindhart, T. D., Lerman, M. and Colburn, N. H.: Life span extension of basal cell nevus syndrome fibroblasts by v-myc or mouse pro gene transfer. Int. J. Cancer (In Press)

Patents:

None

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01CP05383-03 LVC

PERIOD COVERED

October 1, 1985 to September 30, 1986

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Membrane Signal Transduction in Tumor Promotion

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Nancy H. Colburn Chief, Cell Biology Section LVC NCI

Others:	Bonita M. Smith	Guest Researcher	LVC	NCI
	Koichi Hirano	Guest Researcher	LVC	NCI
	John Seed	Guest Researcher	LVC	NCI
	Howard Young	Expert	LMI	NCI
	Kioshi Takahashi	Visiting Fellow	LCC	NCI
	Ursula Heine	Research Microbiologist	LCC	NCI

COOPERATING UNITS (if any)

Inst. Med. Sci., Univ. of Tokyo, Tokyo, Japan (T. Kuroki); Swiss Inst. for Exp. Cancer Res., Lausanne, Switzerland (P. Cerutti); Program Resources, Inc., Frederick, MD (M. Zweig, T. Wood)

LAB/BRANCH

Laboratory of Viral Carcinogenesis

SECTION

Cell Biology Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701-1013

TOTAL MAN-YEARS:

3.5

PROFESSIONAL:

2.1

OTHER:

1.4

CHECK APPROPRIATE BOX(ES)

- (a) Human subjects (b) Human tissues (c) Neither
- (a1) Minors
- (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The goal of these studies is to determine the required biochemical events that occur between tumor promoter-receptor interaction and the activation of effectors of neoplastic transformation. Candidate second messengers include protein phosphorylation, reactive oxygen generation, and calcium mobilization. Both activation of protein kinase C (PKC) and the subsequent loss of PKC activity may be on the signal transduction pathway for TPA-promoted transformation. Pharmacological analogs of calcium, the lanthanides promote neoplastic transformation in JB6 cells by a PKC-independent pathway. The lanthanides, like phorbol esters, induce transformation in (activated) pro-1- or pro-2-transfected P- cells. This indicates that tumor promoters can collaborate with activated pro genes to bring about neoplastic transformation by either PKC-dependent or PKC-independent pathways. The synthesis of nuclear proteins of 15 and 16 kd is TPA inducible in P+ but not in P- cells, an event that may account, in part, for the promotion sensitivity of P+ cells. Finally, P+ and P- cells differ in a transient, TPA-stimulated focus-associated expression of cellular P21 H-ras and an irreversible change in actin configuration, suggesting a possible collaboration of cytoskeletal, cytoplasmic and nuclear proteins with pro genes to bring about transformation.

PROJECT DESCRIPTIONNames, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Nancy H. Colburn	Chief, Cell Biology Section	LVC	NCI
Bonita M. Smith	Guest Researcher	LVC	NCI
Koichi Hirano	Guest Researcher	LVC	NCI
John Seed	Guest Researcher	LVC	NCI
Howard Young	Expert	LMI	NCI
Kioshi Takahashi	Visiting Fellow	LCC	NCI
Ursula Heine	Research Microbiologist	LCC	NCI

Objectives:

To determine the required biochemical events that occur between the interaction of tumor promoters with the plasma membrane and the activation of intracellular effectors of neoplastic transformation. In the case of phorbol diester tumor promoters, a major aim is to identify promotion-relevant events that are closely coupled to phorbol ester receptor binding. Candidates for such second messengers or signal transduction events include protein kinase C-catalyzed protein phosphorylation, reactive oxygen generation and calcium mobilization. Nucleotide sequences in pro genes that constitute recognition signals for 12-O-tetradecanoylphorbol-13-acetate (TPA) and other tumor promoters will be sought. An overall aim is to understand the regulatory interactions between such signal sequences and signal transducers such as C kinase and active oxygen.

Methods Employed:

(1) Assay of calcium-dependent phospholipid-dependent protein kinase (C kinase) activity; (2) assay of the effects of TPA on rate of synthesis and phosphorylation of proteins in intact promotion-sensitive (P⁺) or -resistant (P⁻) cells; (3) immunoprecipitation and Western blotting with antisera to pro-1 peptides or to pro-1 protein synthesized using an expression vector; (4) analysis of pro-1 or pro-2 promoter region by CAT assays and other assays of transcriptional competence; (5) assay of the effects of various modulators of reactive oxygen on promotion of neoplastic transformation (anchorage-independence) by TPA; and (6) assay of the effects of these modulators on the expression of anchorage-independence by tumor cells.

Major Findings:

1. Protein phosphorylation catalyzed by the phorbol ester receptor calcium and phospholipid-dependent protein kinase C. A sustained decrease in cellular levels of protein kinase C may act as part of the signal for promotion of neoplastic transformation. Although C kinase is initially activated by TPA treatment of either P⁺ or P⁻ cells, prolonged exposure of JB6 cells to tumor promoting phorbol esters subsequently produces a dramatic decrease in protein kinase C activity, as measured by calcium and phospholipid-dependent phosphorylation of both endogenous (JB6 cell protein) and exogenous (histone) substrates. After 1 hour of TPA treatment, PKC activity is decreased by 75%, and by 20 hours, activity is not

detectable in any cell fraction. Pharmacological analogs of calcium, the lanthanides, which also promote neoplastic transformation of JB6 cells, activate protein kinase C-catalyzed phosphorylation of the same set of endogenous JB6 cell substrates as calcium. The lanthanides will substitute for calcium in activating calcium- and phospholipid-dependent protein kinase C extracted from JB6 cells. Lanthanides and calcium both support phosphorylation of the same endogenous JB6 cell phosphoproteins, as well as phosphorylation of exogenous (histone) substrates. The mechanism of lanthanide promotion of transformation involves events distal to activation of protein kinase C. Although lanthanides activate protein kinase C in cell lysates, lanthanum fails to activate protein kinase C in intact cells. In contrast to TPA, lanthanum fails to (i) enhance phosphorylation of an 80 Kd protein, an event thought to be indicative of activated PKC; (ii) cause disappearance of PKC activity after prolonged exposure; (iii) produce EGF receptor down regulation with the same magnitude, time course and calcium dependency as TPA. This event is dependent on PKC-catalyzed phosphorylation of EGF receptors; and (iv) induce gamma-interferon in mouse lymphocytes transfected with a cloned human gamma-interferon gene. The extracellular calcium previously found to be required for TPA-induced promotion of transformation in JB6 cells is not essential for activation of the calcium-dependent phorbol ester receptor protein kinase C. EGF receptor down regulation, an event dependent on PKC-catalyzed phosphorylation of EGF receptors, occurs within 30 minutes after exposure of cells to TPA. The time course of inhibition is calcium-independent; the degree of inhibition is identical in calcium-containing (1.6 mM) or calcium-depleted (0.06 mM) medium. The signal for activation of the promotion sensitivity (pro) genes can occur by a mechanism other than activation of the phorbol ester receptor protein kinase C. Lanthanides are strong transformation promoters and do not activate protein kinase C in intact JB6 cells, yet lanthanides and TPA induce equivalent numbers of anchorage-independent colonies in promotion-resistant JB6 cells transfected with active promotion sensitivity genes pro-1 or pro-2.

2. Other signal transduction events. A 21 Kd protein kinase C substrate may be a convergent step on the transformation promotion pathway of TPA and lanthanides. A 23 Kd molecular weight protein kinase C substrate exhibits an altered mobility in SDS polyacrylamide gels in the presence of lanthanum. The effect is still observable if lanthanum is added after SDS and boiling, indicating that the lanthanide effect is probably not dependent on activation of PKC or any other enzyme. The P⁺ and P⁻ phenotypes can be distinguished by (i) P⁺ specific, TPA-induced synthesis of two proteins, and (ii) a P⁺ specific higher basal rate of synthesis of a third protein, events which may account, in part, for the promotion sensitivity of the P⁺ cells. In P⁺ but not P⁻ cell lines, TPA induces the synthesis of two proteins of molecular weight 15 and 16 Kd, with maximal induction observable at 20 hours. These proteins are localized in the nucleus. P⁺ lines also show a constitutively higher rate of synthesis of a 50 Kd protein. Poly ADP-ribosylation may be required for expression of the neoplastic phenotype. 3-aminobenzamide inhibits TPA-induced poly ADP-ribosylation in both P⁺ and P⁻ cell lines in a dose- (1 to 10 mM) and time- (3 to 7 hours) dependent manner and inhibits expression of anchorage-independent colony formation in transformed cells. The calcium-resistant phenotype and the presence of an activated pro gene are not sufficient for conferring sensitivity to promotion of neoplastic transformation. The LC7 and 308 cell lines of H. Hennings (putatively chemically initiated but not promoted) and the JB6 pre promotable cells (spontaneously initiated) are resistant to calcium-induced terminal differentiation but are not

sensitive to TPA-induced neoplastic transformation. Transfection of the active promotion sensitivity genes pro-1 and pro-2 does not confer promotion sensitivity on these cells. Acquisition of an activated pro gene by promotion resistant cells confers a cross sensitivity to other promoters and inhibitors of promotion. Promotion-resistant cells transfected with plasmids containing active pro genes from P⁺ DNA were induced by TPA to anchorage-independent growth in agar. Colonies were cloned from agar to select for cells that had acquired the activated pro gene. When exposed to a series of promoters and inhibitors of promotion, these transfected cells responded similarly to parental P⁺ cells. Lanthanides induced colonies in transfectants with a frequency similar to that of P⁺, not P⁻, cells. The response to TPA of P⁺ cells differs from that of P⁻ cells in showing (i) a transient expression of cellular H-ras p21 associated with focus formation and (ii) persisting changes in actin and vinculin configuration as assayed by fluorescent monoclonal antibodies. Thus, expression of cellular p21 and cytoskeletal proteins may be cooperating with pro genes to bring about neoplastic transformation. The tumor promoter may directly induce expression of some of the cooperating genes. Some of the genes may be expressed transiently, others constitutively, once triggered.

Publications:

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Patents:

None

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01CP05384-03 LVC

PERIOD COVERED

October 1, 1985 to September 30, 1986

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Genetic Analysis of Human Cellular Genes in Neoplastic Transformation

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Stephen J. O'Brien Chief LVC NCI

Others: Janice S. Martenson Microbiologist LVC NCI
 Mary A. Eichelberger Microbiologist LVC NCI
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COOPERATING UNITS (if any) LBI, Fred., MD (G. Vande Woude, M. Cohen, M. Barbacid, E. Brownell);
 USUHS, Beth., MD (E. Chang); CHB, NHLBI, NIH, Beth., MD (N. Anagnou, A. Nienhuis); LMM,
 NIAID, NIH, Beth., MD (M. Martin); Johns Hopkins Hosp., Balt., MD (K. Smith); H&W
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Genetics Section

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TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.5	0.9	0.6

CHECK APPROPRIATE BOX(ES)

- (a) Human subjects (b) Human tissues (c) Neither
 (a1) Minors
 (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The cumulative techniques of cell genetics, molecular biology, linkage analysis and in situ hybridization has resulted in the identification and characterization of over 1500 human loci, a value which now exceeds the number of genes mapped in Drosophila. We have concentrated our efforts on somatic cell hybrid panels and in situ hybridizations to genes related to neoplastic processes including (1) cellular proto-oncogenes, (2) growth factors, (3) growth factors and receptors, (4) endogenous retroviral families, (5) integration sites for retroviruses, and (6) restriction genes that delimit retrovirus replication in mammals. Within the last few years, the human gene map has experienced a large increase in the number of neoplasia loci that have been mapped to specific chromosomal positions. Of the 35 specific human loci that have been chromosomally mapped to date, 13 (40%) have been assigned by the Genetics Section scientists and their collaborators. This year, we have concentrated on understanding the genomic organization of several genes: rel, ets, trk, tpr, fms, met and endogenous retroviral families. Three of these genes, ets, met-tpr and trk, were found to be composite genes derived from the fusion of chromosomally disparate functional loci. Truncation of these cellular genes in a variety of human neoplasias, as well as in certain nonneoplastic pathologies (e.g., ets-2 in Down's syndrome or met in cystic fibrosis) which were suggested by their chromosomal position, are under investigation. The emerging gene map neoplasia-associated loci continues to provide an unprecedented opportunity for molecular genetic analysis of the initiation and progression of neoplastic processes.

PROJECT DESCRIPTIONNames, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Stephen J. O'Brien	Chief	LVC	NCI
Janice S. Martenson	Microbiologist	LVC	NCI
Mary A. Eichelberger	Microbiologist	LVC	NCI
Takis S. Papas	Chief	LMO	NCI
William S. Modi	Staff Fellow	LVC	NCI
Dennis K. Watson	Senior Staff Fellow	LMO	NCI

Objectives:

1. The augmentation of the human gene map with loci that have direct or indirect connections to the processes of neoplastic transformation in man or other vertebrate model systems. The specific genes under study fall into six general groups: (1) cellular proto-oncogene loci, (2) growth factors, (3) receptors for growth factors and retroviruses, (4) endogenous cellular DNA sequences homologous to retroviral RNA genomes, (5) chromosomal integration sites for chronic transforming retroviruses and (6) restriction genes that delimit retroviral replication.
2. Genetic analysis of cooperative and sequential gene actions in the neoplastic processes and in virogene expression. This consideration involves application of the principles and techniques of microbial genetics to cultured mammalian cells.
3. The development of new approaches to the understanding of genetic control of carcinogenesis. This goal involves the identification and characterization of genetic targets (cellular genes) of carcinogenesis.
4. The resolution of the sequence and tissue specificities of distinct cellular genes in different human malignancies. Thus, neoplastic pathways of genetic networks can be dissected by genetic analysis in protocols reminiscent of dissection of metabolic enzyme pathways in early microbial studies.

Methods Employed:

The following techniques were employed: (1) somatic cell hybridization and various gene transfer procedures; (2) isoenzyme electrophoresis and histochemical development on starch, acrylamide and cellulose acetate; (3) cytological chromosome banding procedures (G and Q, G-11) for specific chromosome identification; (4) virological procedures including radioimmunoassay (RIA), reverse transcriptase assay and viral cloning; (5) immunological assays including RIA, cytotoxicity, fluorescent antibody procedures and monoclonal antibody preparation in murine hybridomas; (6) molecular biology techniques including cDNA transcription in vitro, solution hybridization, visualization of restricted DNA by the techniques of Southern following agarose electrophoresis and molecular cloning of eukaryotic genes; and (7) in situ hybridization to metaphase chromosomes.

Major Findings:

1. Gene mapping and analysis of human proto-oncogenes. Cellular transforming genes have developed an established role in the ontogeny of human neoplasias. They have been associated in human and animal neoplasias in a number of ways including: (1) transduction of proto-onc genes by retroviruses, (2) integration of nontransforming retroviruses adjacent to cellular onc genes and alteration of their expression, (3) specific transformation of murine 3T3 upon transfection with genomic DNA from human tumors, (4) chromosomal translocation of proto-onc gene adjacent to regulatory elements that alter their transcription, (5) genetic amplification of proto-oncogene segments in certain tumors, and (6) point mutation of nonneoplastic gene homologues. Because of their suspected importance in human carcinogenesis, nearly all human homologues have been chromosomally assigned. Our laboratory has reported the chromosomal assignment (using somatic cell hybrid panels and *in situ* hybridization) of 13 of the approximately 30 plus proto-oncogene loci thus far localized in man. These include trk, fms, tpr, met, trk, Ha-ras-1, Ha-ras-2, Ki-ras-1, Ki-ras-2, raf-1, raf-2, rel, ets-2 and ets-2. The regional association of each of these loci has been determined and their position with relationship to adjacent loci and with respect to nonspecific chromosomal rearrangement in human tumors has been evaluated. In combination with results of oncogenes and other classes of neoplasia-related loci from our laboratory and others, the oncogene families represent a rich opportunity for dissecting the series of genetic events that we term "neoplastic transformation."

2. Genetic demonstration that the human met is a translocation mediated fusion of two genes, met and tpr. The met oncogene, isolated from the tumorigenic, N-methyl-N'-nitrosoguanidine (MNNG)-treated human osteogenic sarcoma cell line MNNG-HOS, is 25 to 35 kilobases (kb) in size and is related to the tyrosine kinase family of genes. Genomic probes from this locus recognize two distinct RNA transcripts which are independently expressed in a cell-type specific fashion; one is a 10.0-kb RNA that is expressed in all human cell lines we have tested, while the other is a 9.0-kb RNA that (with one exception) is not detected in cells of hematopoietic lineage. The met proto-oncogene locus which expresses the 9.0-kb RNA was mapped to human chromosome 7q21-31. The 10.0-kb RNA locus, which we refer to as tpr (translocated promote region), maps to human chromosome 1. Thus, we conclude that the chemical transformation of HOS cells involved the genetic fusion of two chromosomally disparate loci, met and tpr, possibly by chromosomal translocation, which produced the functional hybrid met oncogene. Both MNNG-HOS cells and NIH 3T3 cells transformed by met express a novel 5.0-kb RNA species which is a composite transcript of both tpr and met proto-oncogene sequences. The gene fusion event apparently activates the met oncogene via expression of a hybrid DNA transcript with 5' sequences derived from the tpr locus (normally on chromosome 1) and 3' sequences containing the kinase homology, from the met locus normally located on chromosome 7.

3. Implication of the c-fms gene in the etiology of the 5q- refractory anemia syndrome in man. The c-fms proto-oncogene was shown to be expressed in normal human bone marrow and in differentiated blood cells, suggesting that its gene product plays a role in the terminal stages of hematopoietic maturation. The c-fms mRNA was not detected in HL-60 cells, an established promyelocytic line, whereas c-fms expression appeared 48 hours after induction with phorbol ester when

most cells had differentiated into adherent, post-mitotic macrophages. An acquired deletion of chromosome 5 (5q⁻) in bone marrow cells, the human 5q⁻ syndrome, was associated with abnormalities in blood cell production. The normal 5 and 5q⁻ chromosomes were individually segregated by construction of cell hybrids between bone marrow cells and rodent cells. A selective system was used that requires retention of the structural gene for dihydrofolate reductase, located on human chromosome 5. Analysis of DNA from individual hybrid clones revealed that the 5q⁻ deletion had removed the c-fms gene. These results suggest that hemizy-gosity at the c-fms locus leads to abnormalities in hematopoietic maturation, specifically the 5q syndrome in man.

4. The c-ets homolog of the avian E26 transforming virus is two chromosomally separate transcriptionally active genes (ets-1 and ets-2) in the human genome. Chicken and human DNA related to the ets-region from the transforming gene of avian erythroblastosis virus, E36, was molecularly cloned and shown to be closely related to the viral equivalent by hybridization and partial sequence analysis. The transforming gene of E26 is a tripartite hybrid with the structure Δ gag (1.2-kb, from the viral gag gene)-myb (0.9-kb from the chicken myb gene)-ets (1.6-kb, from the chicken ets gene). Human ets DNA is located on two distinct human chromosomes. The human ets-1 locus on chromosome 11, encodes a single mRNA of 6.8-kb, while the second locus, ets-2 on chromosome 21, encodes three mRNAs of 4.7, 3.2 and 2.7-kb. The ets-related sequences of human DNA on chromosomes 11 and 21 do not overlap. By contrast, the chicken genomic homolog appears to be a single gene containing contiguous ets-1 and ets-2 sequences and is primarily expressed in normal chicken cells as a single 7.5-kb mRNA. We conclude that the ets sequence shared by the virus, the chicken and man is likely to contain two dissociable functional domains, ets-1 and ets-2. Thus, the tripartite transforming gene of E26 includes four distinct domains which may be functionally relevant for transforming function of the virus (Δ gag, myb, ets-1 and ets-2).

5. Dispersion of endogenous retroviral families to multiple chromosomes in man. Three independent laboratories, using molecularly cloned probes, have identified three apparently different families of human DNA sequences that are related to replication-competent retroviruses isolated from other mammals. The groups include (1) a small group distantly related to Mo-MuLV and BaEV, described by M. Cohen et al.; (2) a larger family of 30-50 segments related to Mo-MuLV, described by M. Martin et al.; and (3) a family related to the mouse mammary tumor virus, described by R. Callahan et al. We have collaborated with Dr. M. Cohen on the genetic association of the ERV family and have assigned three members, ERV1, ERV2 and ERV3, to three distinct human chromosomes. One of these, ERV3, is apparently transcriptionally active because specific transcripts can be detected using Northern analysis. The hybrid panels have also been used to examine two distinct families described by M. Martin and his collaborators. The first is a truncated group that lacks LTR sequences. The second is a full-length viral family with approximately 30 members. Both families have been found to be dispersed to multiple chromosomes as well. The chromosomal positions of several of these have been determined using specific env probes, as well as cellular flanking DNA as probes, in the hybrid panel. The genetic associations of these endogenous retroviral sequences and proto-oncogenes or other cis-associated neoplastic loci are under investigation.

6. Molecular cloning and genetic analysis of the human structural gene for endothelial cell growth factor (ECGF). Several endothelial cell polypeptide mitogens that have been described probably play a role in blood vessel homeostasis. Two overlapping cDNA clones encoding human ECGF have been isolated from a human brain stem cDNA library. Southern blot analysis suggested there is a single copy of the ECGF gene which was mapped to human chromosome 5 at bands 5q31.3-33.2. A 4.8-kb messenger RNA (mRNA) was present in human brain stem mRNA. The complete amino acid sequence of human ECGF has been deduced from the nucleic acid sequence of these clones and encompasses all the well-characterized acidic endothelial cell polypeptide mitogens described by several laboratories. The ECGF-encoding open reading frame is flanked by translation stop codons, and provides no signal peptide or internal hydrophobic domain for the secretion of ECGF. This property is shared by human interleukin 1, which is approximately 30 percent homologous to ECGF. The similarities between ECGF and interleukin 1 are discussed in terms of vessel wall homeostasis.

Publications:

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Park, M., Dean, M., Cooper, C. S., Schmidt, M., O'Brien, S. J., Blair, D. G. and Vande Woude, G. F.: Mechanism of met oncogene activation. Cell (In Press)

Surti, U., Szulman, A. E., Wagner, K., Leppert, M., White, R. and O'Brien, S. J.: Tetraploid partial hydatichform moles: Two cases with a triple paternal contribution and a 92,XXY karyotype. Hum. Genet. 72: 15-21, 1986.

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Patents:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05385-03 LVC

PERIOD COVERED

October 1, 1985 to September 30, 1986

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Molecular Genetic Analysis of Feline Cellular Genes: A Comparative Approach

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	Stephen J. O'Brien	Chief	LVC	NCI
Others:	William S. Modi	Staff Fellow	LVC	NCI
	David Derse	Guest Researcher	LVC	NCI
	Cheryl A. Winkler	Biologist	LVC	NCI
	Naoya Yuhki	Visiting Fellow	LVC	NCI
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	Janice S. Martenson	Microbiologist	LVC	NCI
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LAB/BRANCH

Laboratory of Viral Carcinogenesis

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Genetics Section

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TOTAL MAN-YEARS:

3.6

PROFESSIONAL:

2.6

OTHER:

1.0

CHECK APPROPRIATE BOX(ES)

- (a) Human subjects (b) Human tissues (c) Neither
- (a1) Minors
- (a2) Interviews

SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

The development of the domestic cat as an animal model for genetic analysis began with the construction of a genetic map which now consists of over 50 loci. A striking linkage homology of the human and feline gene maps was confirmed by cytological procedures and today over 25% of the human genome can be aligned band for band with the feline karyotype. Typing sera for the feline major histocompatibility complex were generated by allogenic skin grafting and serological reagents were evaluated by population cluster analysis and by immunoprecipitation of feline lymphocyte antigens. The molecular organization of the feline MHC complex was derived using homologous gene clones and compared to the human and murine MHC clusters. Evolution of the proto-oncogene family of mammals has been studied by mapping these genes in cat, mouse and man, and reconstructing their natural history. Two endogenous retroviral families (RD-114 and FeLV) have been studied from a molecular perspective and appear to represent vestiges of ancient infections and gene amplification in the Felidae. An evolutionary tree of 37 species of the cat family has been constructed using two molecular measures of genetic distance.

PROJECT DESCRIPTIONNames, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Stephen J. O'Brien	Chief	LVC	NCI
William S. Modi	Staff Fellow	LVC	NCI
David Derse	Guest Researcher	LVC	NCI
Cheryl A. Winkler	Biologist	LVC	NCI
Naoya Yuhki	Visiting Fellow	LVC	NCI
Stanley Cevario	Microbiologist	LVC	NCI
Janice S. Martenson	Microbiologist	LVC	NCI
Mary A. Eichelberger	Microbiologist	LVC	NCI

Objectives:

1. The development and expansion of the genetic map of the domestic cat (Felis catus) with particular emphasis on genes of the immune system that have an oncogenic role in leukemia and lymphomas and that control developmental processes. The specific classes of genes under study include (1) endogenous cellular DNA sequences homologous to retroviral RNA; (2) chromosomal integration sites for exogenous retroviral insertion and persistence; (3) receptors on cell membranes that interact with viral glycoproteins to determine cell-species compatibility and viral host range; (4) restriction genes that delimit virus replication in various animal species; (5) cellular transforming onc genes; (6) enzyme structural genes; and (7) cell surface antigens, including antigens homologous to the major histocompatibility complex (MHC) of other mammalian species.
2. Comparative linkage analysis of the feline gene map with two other major mammalian gene maps, human and mouse, for reconstruction of natural history of chromosomal rearrangements of the three mammalian orders.
3. Development of serological and cellular immunological reagents for analysis of feline lymphoid and myeloid-cell derivatives with special emphasis on correlation of function of T- and B-cell subsets.
4. Molecular and genetic analysis of endogenous feline retroviral families (specifically RD-114 and FeLV).
5. Development of feline embryo manipulation for gene therapy and production of transgenic cats.

Methods Employed:

The following techniques were employed: (1) somatic cell hybridization and various gene transfer procedures; (2) isoenzyme electrophoresis and histochemical development on starch, acrylamide and cellulose acetate; (3) cytological chromosome banding procedures (G and Q, G-11) for specific chromosome identification; (4) virological procedures, including radioimmunoassay (RIA), reverse transcriptase assay and viral cloning; (5) immunological assays, including RIA, cytotoxicity assays, fluorescent antibody procedures, immunoprecipitation,

and monoclonal antibody preparation in murine hybridomas; (6) molecular biology techniques, including cDNA transcription *in vitro*, molecular cloning, gene splicing, DNA and RNA blotting; and (7) in situ DNA hybridization to metaphase chromosomes.

Major Findings:

1. Conserved linkage homology between the human and feline gene maps is evidenced in over 50 homologous loci. Development of the feline genetic map in our laboratory revealed that 50 biochemical loci assigned to 16 of the 19 cat chromosomes were particularly homologous in linkage to the human map using the same loci. Using high resolution G-trypsin banding (1000 band level of resolution), we were able to demonstrate that 20-25% of the human genome could be aligned band for band to homologous regions. Linkage homologies in other regions were characterized by small intrachromosomal rearrangements. The extensive advances in comparative syntenic and cytogenetic analyses have been utilized to partially reconstruct the chromosomal breaks that have occurred during 100 million years of mammalian evolution. The conservation of linkage association is also considered in identifying mammalian genes (such as retroviruses or controlling elements) capable of transposition during species transitions.
2. Chromosomal linkage of beta-globin and albino (siamese) in the domestic cat revealed an extensively conserved autosomal synteny in mammals and predicts the precise chromosomal position of heretofore unmapped human albino gene. Siamese cats are homozygous for the recessive c^s allele of the color (albino) locus. The c locus was shown, by backcross analysis, to be linked to the beta-hemoglobin (HBB) locus in the cat at a distance of approximately eight centi-Morgans. The HBB locus and, by inference, the c locus were assigned to feline chromosome D1, by analysis of genomic DNAs from a panel of rodent x cat somatic cell hybrids with a molecular clone of the human beta-globin locus. Evolutionary conservation of the syntenic homology of feline chromosome D1 and human chromosome 11 is extensive. Comparison of high resolution G-trypsin banded preparations of the two chromosomes permitted cytological alignment of the long arm of the conserved chromosomes providing that a minimum of one paracentric inversion is hypothesized. The placement of the albino locus on conserved syntenic groups of several markers (HBB, HRAS, LDHA) in both cat and mouse strongly indicates the conservative placement of the as yet unmapped human albino locus in the homologous syntenic group on human chromosome 11p.
3. The viral ets proto-oncogene consists of dual coding domains which were homologous to chromosomally distinct and transcriptionally active proto-oncogenes in cats, mice and humans. The mammalian proto-oncogene homologue of the avian v-ets sequence from the E26 retrovirus consists of two sequentially distinct domains located on different chromosomes. Using somatic cell hybrid panels, the mammalian homologue of the 5' v-ets-domain (ets-1) was mapped to chromosome 11 in man, to chromosome 9 in mouse, and to chromosome D1 in the domestic cat. The mammalian homologue of the 3' v-ets domain (ets-2) was similarly mapped to human chromosome 21, to mouse chromosome 16, and to feline chromosome C2. Both proto-oncogenes fell in syntenic groups of homologous-linked loci which were conserved among the three species. The occurrence of two distinct functional proto-oncogenes and their conservation of linkage positions in the three mammalian orders indicate

that these two genes have been separate since before the evolutionary divergence of mammals.

4. Definition of FLA, the feline MHC. The major histocompatibility complex responsible for synthesis of antigens that play a key role in graft rejection and in T-cell communication (MHC restriction, associative recognition) have been described in several laboratory animals (and man) with the glaring exception of the domestic cat. The participation of the MHC in the development of the immune response, resistance to leukemias, susceptibility to various diseases, and transplant rejection makes this locus a high priority in the study of feline genetics. We have initiated a closed breeding colony of 100 cats at the NIH Animal Center in Poolesville, MD, for a number of genetic and physiological experiments (see Project Number Z01CP05389-03 LVC). Reciprocal, split-thickness skin grafts were surgically exchanged between over 120 animals over the past four years.

Approximately 75 cats rejected the grafts acutely (representing a difference(s) at the MHC), while 25 cats rejected chronically (representing identity at the MHC and differences at minor histocompatibility loci). Fifteen of the 75 positive cats produced cytotoxic antisera as measured with a two-stage cytotoxic antibody assay. These sera were analyzed by typing over 300 cats from four colonies—using pedigree and "cluster" analyses. The derived sera are highly representative because every cat tested was positive with at least one of the alloantisera. Pedigree and cluster analyses have resulted in the definition of specific MHC epitopes and haplotypes in natural populations. Immunoprecipitation experiments revealed that several sera recognized class I determinants (MW=45,000), while others recognized class II molecules (MW=32,000).

The molecular structure of the feline MHC has been examined with specific molecular clones of human and murine class I and class II genes. The number of feline class I genes is estimated at between 10-15 copies/haploid genome and class II genes 1-2 α and 1-2 β subunit loci. Both class I and class II sequence have been assigned to feline chromosome B2 which contains several gene markers homologous to human chromosome 6. Specific feline cDNA clones of MHC class I genes have been isolated and are being characterized. The development and characterization of both immunologic and molecular MHC reagents represent a significant advance in the immunobiology of the domestic cat model.

5. Molecular genetic characterization of the RD-114 family of endogenous retroviral genes in the cat. RD-114 is a replication-complement, xenotropic retrovirus that is homologous to a family of moderately repetitive DNA sequences present in approximately 20 copies in the normal cellular genome of domestic cats. In order to examine the extent and character of genomic divergence of the RD-114 gene family, as well as to assess their positional association within the cat genome, we have prepared a series of molecular clones of endogenous RD-114 DNA segments from a genomic library of cat cellular DNA. The endogenous sequences analyzed were similar to each other in that they were colinear with RD-114 proviral DNA, were bound by long terminal redundancies and conserved many restriction sites in the gag and pol regions. However, the env regions of many of the sequences examined were substantially deleted. The endogenous RD-114 sequences each had a distinct cellular flank and were dispersed on multiple feline chromosomes. The chromosomal locations of four RD-114 virogenes (RDV1-4) were

determined using a panel of rodent x cat somatic cell hybrids. One of these, RDV1, is apparently a single locus on feline chromosome B3, which is inducible for replication-complement RD-114 virus.

Publications:

Berman, E. J., Nash, W. G., Seuanez, H. N. and O'Brien, S. J.: Chromosomal mapping of enzyme loci in the domestic cat: GSR to C2, ADA and ITPA to A3, and LDHA-ACP2 to D1. Cytogenet. Cell Genet. 41: 114-120, 1986.

Collier, G. E. and O'Brien, S. J.: A molecular phylogeny of the Felidae: Immunological distance. Evolution 39: 473-487, 1985.

O'Brien, S. J.: Genetic analysis in mammals: Past, present, and future. In Evans, J. W. and Hollaender, A. (Eds.): Proceedings of Genetic Engineering in Animals: An Agricultural Perspective. New York, Plenum Press, 1986, pp, 139-149.

O'Brien, S. J.: Molecular genetics in the domestic cat and its relatives. Trends Genet. 2: 137-142, 1986.

O'Brien, S. J., Haskins, M. E., Winkler, C. A., Nash, W. G. and Patterson, D. F.: Chromosomal mapping of beta-globin and albino loci in the domestic cat reveals an extensively conserved autosomal syntenic group in mammals. J. Hered. (In Press)

O'Brien, S. J., Seuanez, N. H. and Womack, J. E.: On the evolution of genome organization in mammals. In MacIntyre, R. J. (Ed.): Molecular Evolutionary Genetics (Monographs in Evolutionary Biology Series). New York, Plenum Press, 1985, pp. 519-589.

Reeves, R. H., Nash, W. G. and O'Brien, S. J.: Genetic mapping of endogenous RD-114 retroviral sequences of the domestic cat. J. Virol. 56: 303-306, 1985.

Watson, D. K., Smith, M. J., Kozak, C., Reeves, R., Gearhart, J., Numm, M. F., Nash, W., Fowle, J. R., III, Duesberg, P., Papas, T. S. and O'Brien, S. J.: Conserved chromosomal positions of dual domains of the ets proto-oncogene in cats, mice and man. Proc. Natl. Acad. Sci. USA 83: 1792-1796, 1986.

Patents:

None

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01CP05386-03 LVC

PERIOD COVERED

October 1, 1985 to September 30, 1986

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Basic Mechanisms in HTLV-Induced Leukemia and AIDS

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	Stephen J. O'Brien	Chief	LVC	NCI
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Others:	David Derse	Guest Researcher	LVC	NCI
	James W. Casey	Senior Staff Fellow	LVC	NCI
	Robert C. Gallo	Chief	LTCB	NCI

COOPERATING UNITS (if any)

Upstate Medical Center, Syracuse, NY (B. Poiesz); LMM, NIAID, NIH, Bethesda, MD (M. Martin)

LAB/BRANCH

Laboratory of Viral Carcinogenesis

SECTION

Genetics Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701-1013

TOTAL MAN-YEARS:

0.7

PROFESSIONAL:

0.5

OTHER:

0.2

CHECK APPROPRIATE BOX(ES)

- (a) Human subjects (b) Human tissues (c) Neither
- (a1) Minors
- (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

T-cell lymphotropic retroviruses (HTLV-I, II and III) have an affinity of human T lymphocytes and enter the cells by specific interaction with the T-4 receptor. All three types of HTLVs have been transmitted *in vitro*, molecularly cloned and sequenced. Despite these advances, the mechanism by which infection with these viruses results in malignant transformation or immunosuppression remains unknown. We are focusing on basic mechanisms, both on a cellular and molecular level, by which these viruses transform or immunosuppress. In order to address whether HTLV-I may induce transformation through an insertional mutagenesis mechanism, we have utilized somatic cell hybrids constructed between rodent cells and HTLV-I-infected human cell lines to study the processes and consequences of HTLV chromosomal integration. Integration *in vitro* was shown to be a dynamic process and proviral integration apparently occurs at random in the genome. We have also utilized the panel of Hut 102 X Chinese hamster hybrids to demonstrate that the novel Class I antigenic determinants expressed on HTLV-I-infected cells do not result from induction of Class I genes encoded by the cellular MHC locus, but are probably encoded by integrated HTLV-I. The activities of the promoter unit contained within the LTR of both HTLV-I and HTLV-III were examined by transfecting various cells with recombinant plasmids containing the LTR of HTLV-I or HTLV-III linked to the bacterial gene for chloramphenicol acetyltransferase (CAT). We have demonstrated that infected cells contain factors that act in trans on the LTRs of the infecting virus to activate transcription. Two families of endogenous retroviral sequences were shown to be widely dispersed in the human genome using genetic analysis. The sequences are the consequence of an ancient evolutionary gene amplification which resulted in greater than 0.1% being homologous to retroviral sequences.

PROJECT DESCRIPTIONNames, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Stephen J. O'Brien	Chief	LVC	NCI
David Derse	Guest Researcher	LVC	NCI
James W. Casey	Senior Staff Fellow	LVC	NCI
Robert C. Gallo	Chief	LTCB	NCI

Objectives:

1. Determination of the chromosomal integration site(s) of human T-cell lymphotropic retrovirus-I (HTLV-I) in infected leukemic and lymphoma cell lines and fresh tumor tissue. Proviral integration sites will then be compared to the position of certain human loci that are candidates for genetic causes of transformation, including proto-oncogenes, transforming genes active in the DNA transfection assay, genes encoding growth factors or their receptors, as well as translocation breakpoints characteristic of specific neoplasias.
2. Application of somatic cell genetics to the dissection of certain biologic and immunologic phenomena associated with HTLV infection in order to determine their role in the pathogenesis of HTLV-induced neoplasia and immune suppression.
3. Transfection of recombinant plasmids containing HTLV-I and HTLV-III regulatory sequences linked to the chloramphenicol acetyltransferase (CAT) gene in order to elucidate the phenomenon of "trans-activation" observed in HTLV-I-infected cells.
4. Description of the genomic organization of endogenous human retroviral families.

Methods Employed:

The following techniques were employed: (1) somatic cell hybridization, (2) starch gel electrophoresis with histochemical isoenzyme analysis, (3) G-11 chromosome staining and G-trypsin chromosome banding, (4) molecular biologic techniques such as DNA and RNA blotting, (5) radioimmune assays, (6) fluorescent antibody staining and use of the fluorescent-activated cell sorter (FACS), (7) DNA transfection, (8) assays for genomic enhancer elements using the CAT, and (9) molecular cloning and gene splicing.

Major Findings:

1. Chromosome integration of HTLV-I in human tumors is monoclonal, dynamic, and chromosomally dispersed. The progression of HTLV-I proviral integration over a 3-year period of in vitro culture was examined in two human lymphoma lines, Hut 102 and MJ. Using specific HTLV-I molecular clones and a Southern analysis at different cell passages, Hut 102 increased from 2 to 19 integrated proviral integrations while MJ increased to at least 25 different integration by passage 43. During the progress of increased superinfection and novel integration in vitro,

some of the previous proviral integrations were lost from the cultures. The 19 integrations of late passage Hut 102 cells were shown to be dispersed to 19 different human chromosomes by analysis of 34 distinct rodent x Hut 102 somatic cell hybrids which segregated human chromosomes (and included proviral integrations) in different combinations. The two primary integrations in Hut 102 were located on human chromosomes 4 and 20, respectively. A similar pattern of non-specific integration was observed in somatic cell hybrid analysis of the 25 proviral integrations of MJ. The dynamic infection-reintegration process in vitro revealed in these studies may confuse experimental verification of potential cis-acting functions of HTLV-I in the as yet poorly understood mechanism of neoplastic transformation.

2. HTLV-I infected cells express a novel antigen which mimics the HLA class I antigenic specificity. The unscheduled antigenic expression is in addition to and distinct from class I antigens expressed by autologous uninfected cells. We have demonstrated that genomic DNA of infected cells does not contain detectable rearrangements of HLA class I genes nor does it contain specific polymorphic restriction fragments characteristic of the "novel" HLA determinants in normal individuals possessing the HLA genotype. Furthermore, we have shown that the expression of these HLA-related novel antigens in hybrids of HTLV-I-infected cells and hamster cells, as measured by a specific monoclonal HLA antibody, did not segregate with the human HLA locus on chromosome six, but rather assorted with multiple chromosomes which contained integrated HTLV-I proviruses. These results clearly indicate that the expression of novel HLA antigens does not result from induction of class I genes encoded by the cellular MHC locus and support the explanation that the novel HLA antigen expression associated with HTLV-I is determined and possibly encoded by integrated HTLV-I. The physical consequences of novel HLA antigen expression on the host immune response to infection is under investigation.

3. Demonstration of functional activity of the promoter unit contained within the long terminal repeats (LTRs) of HTLV-I and HTLV-III (pHTLV-I-CAT and pHTLV-III-CAT coupled to the bacterial gene for CAT. Comparison of the levels of CAT activity in a panel of various cell types transfected with pHTLV-I-CAT has revealed that all cells productively infected with HTLV-I (but not HTLV-III or bovine leukemia virus, BLV) have high levels of CAT activity. Uninfected cells have low levels of CAT activity. This data indicates that infected cells contain factors that act in trans on the HTLV-I LTR to activate transcription. We have also demonstrated that a B-cell line, HS-1, which contains integrated HTLV-I and is able to transmit the virus in vitro, fails to transactivate the HTLV-I LTR. Examination of several cell lines (both B and T) infected in vitro by cocultivation with HS-1 revealed a spectrum of CAT activity that parallels, precisely, the level of HTLV-I specific mRNA in each cell line. This data suggests that the cellular environment plays a critical role in the regulation of both HTLV transcription and transactivation. Similarly, all cells productively infected with HTLV-III (but not HTLV-I or BLV) yield high levels of CAT activity after transfection with pHTLV-III-CAT. We have demonstrated that a molecular clone of HTLV-III, HXB2, will result in significant levels of CAT activity within 48 hours following co-transfection with pHTLV-III-CAT in the T-cell line, H9. We are currently examining deletions of the HXB2 clone in co-transfection experiments in order to precisely determine the region of the viral genome required for trans-activation. Taken as a whole, the data indicate that cells infected with HTLV-I or HTLV-III synthesize factors that enhance

viral transcription. A key issue, and one on which we are now focussing, is whether these transacting factors are directly encoded by the virus, the cellular genome or both acting in concert.

4. The human genome contains a large amount of endogenous retroviral DNA segments which are the result of an ancient amplification event. The human genome contains two distinct families of endogenous retroviral sequences: a full-length retroviral family (FRV) and truncated retroviral family (TRV). The number of copies of these sequences approaches 50 and evidence of coordinate amplification of the TRV sequences and their flanking cellular DNA prompted a study of the FRV family. Analysis of several molecular clones of the FRV family indicates that this family also is characterized by coordinate amplification of viral and flanking DNA sequences. The appearance of similar restriction fragments for internal and junction fragments in man and chimpanzee DNAs suggests that the original FRV amplification occurred prior to the evolutionary divergence of the species. Southern analysis of a panel of 60 rodent X human somatic cell hybrids, using FRV- and TRV-specific molecular probes, demonstrated resolution and independent chromosomal segregation of 17 FRV and 18 TRV segments. The results indicate that the 35 to 45 endogenous retroviral sequences have been dispersed by an ancient amplification event to multiple chromosomal positions in man. Three FRV and five TRV viral sequences were chromosomally mapped by their concordant appearance with specific human chromosomes in the hybrid panel.

Publications:

Seigel, L. J., Nash, W. G., Poesz, B. J., Moore, J. L., Wong-Staal, F., Gallo, R. C. and O'Brien, S. J.: Human T-cell leukemia/lymphoma virus type-I integration in infected cell lines: A genetic analysis. Virology (In Press)

Seigel, L. J., Ratner, L., Josephs, S. F., Derse, D., Feinberg, M., Reyes, G., O'Brien, S. J. and Wong-Staal, F.: Transactivation induced by human T lymphotropic virus type III (HTLV-III) maps to a viral sequence encoding 58 amino acids and lacks tissue specificity. Virology 148: 226-231, 1986.

Steele, P. E., Martin, M. A., Rabson, A. B., Bryan, T. and O'Brien, S. J.: Amplification and chromosomal dispersion of human endogenous retroviral sequences. Mol. Cell. Biol. (In Press)

Patents:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05388-03 LVC

PERIOD COVERED

October 1, 1985 to September 30, 1986

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Molecular Basis of Induction of Neoplasia by Feline Leukemia Virus

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: James W. Casey Senior Staff Fellow LVC NCI

Others: None

COOPERATING UNITS (if any)

Program Resources, Inc., Frederick, MD (M. Braun, M. Gonda)

LAB/BRANCH

Laboratory of Viral Carcinogenesis

SECTION

Genetics Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701-1013

TOTAL MAN-YEARS:

0.9

PROFESSIONAL:

0.3

OTHER:

0.6

CHECK APPROPRIATE BOX(ES)

- (a) Human subjects (b) Human tissues (c) Neither
 (a1) Minors
 (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Transcriptional regulation of the type C retrovirus, feline leukemia virus (FeLV), depends upon the interaction of cellular encoded factors with specific sequences located in the U3 portion of the long terminal repeat (LTR). We have previously isolated a defective FeLV provirus from a T-cell lymphosarcoma which had transduced the proto-oncogene, *c-myc*. The transduced feline, *v-myc*, was shown to be identical to *c-myc* in nucleotide sequences, substantiating that amino acid substitution was not a requirement for transforming function. These data suggest that the level of transcripts emanating from the FeLV-*myc* provirus is a major determinant of transformation. To quantitate the promoter strength of the FeLV-*myc* LTR, the chloramphenicol acetyl transferase (CAT) assay was employed and data obtained were compared to the Garder Arnstein (GA) FeLV promoter. The results show that the FeLV-*myc* promoter is six times stronger than the GA FeLV promoter in T-cells, the tumor cell type from which FeLV-*myc* was cloned. In B-cells or fibroblasts, the FeLV-*myc* and GA FeLV promoters are equal in transcriptional strength. Two major differences are apparent in the FeLV-*myc* LTR compared with the GA LTR, a 44 base pair (bp) duplication and 7 nucleotide substitutions in U3. To assess the value of these alterations in conferring T-cell preference to the FeLV-*myc* promoter, *in vitro* recombinations were made between the FeLV-*myc* and GA LTR. Each construct was measured for T-cell preference using the CAT assay. The enhancer, a 44 bp duplication, accounts for 30% of this T-cell preference, while 5' and 3' nucleotide substitutions appear to be the major determinants.

PROJECT DESCRIPTIONNames, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

James W. Casey	Senior Staff Fellow	LVC	NCI
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Objectives:

The feline leukemia viruses are proving to be a unique system to evaluate the regulation of transduced oncogenes and to study eukaryotic gene expression. The objectives of our current research are to isolate and define the T-cell specific responsive elements in retroviral promoters. Once this information is obtained, the isolation of cellular encoded transcriptional factors can be initiated.

Methods Employed:

The following techniques were employed: (1) genomic molecular cloning in factors, (2) DNA-mediated transfection, (3) cDNA library construction, (4) SV40 chloramphenicol acetyl transferase (CAT) assays to measure promoter/enhancer activity, (5) DNA sequence analysis using the Sanger shotgun technique and (6) Southern blotting and mapping of eukaryotic genes.

Major Findings:1. The FeLV myc LTR is T-cell tropic.

The functional analysis of the FeLV-myc LTR was evaluated by linking the LTR to the bacterial gene which encodes the enzyme CAT, transfecting this construct onto different cell lines and measuring levels of enzyme (CAT) activity. Direct comparison of the transcriptional activity and cell type specificity of the FeLV-myc LTR was made using the GA FeLV LTR as a control. The phenomenon that we observed using murine (BW5147) or human (H-9) T-cells is that the FeLV-myc LTR has a high T-cell preference; namely, the FeLV-myc LTR is five to six times more active in T-cell than the GA LTR. Furthermore, this T-cell preference displayed by the FeLV-myc LTR is conserved functionally across species from mouse to human. Since the transcriptional preferences is confined to T-cells and not B-cells or fibroblasts, the data suggest that T-cells contain a unique factor that recognizes the FeLV-myc LTR.

2. Identification of the T-cell responsive elements in the FeLV-myc LTR.

The complete nucleotide sequence of the FeLV-myc LTR and the GA FeLV-LTR have been determined. The most striking difference between these two promoter units is a 44 base pair (bp) duplication in FeLV-myc, which contains a core enhancer sequence. Also, seven nucleotide substitutions have been identified in the 5' portion of FeLV-myc. Since these are the only alterations between these two FeLV promoters, we propose that the T-cell preference displayed by the FeLV-myc LTR must be due to all or some of these changes. We have removed one copy of the FeLV-myc 44 bp duplication and inserted this segment into the GA FeLV LTR. Measurements of transcriptional activity driven by these two recombinants show that 30% of the

T-cell preference can be accounted for by the duplication. The 5' portion of each promoter, from the beginning of the promoter to the center of the enhancer, was exchanged between FeLV-myc and GA FeLV. Results of these data show that four nucleotide substitutions account for approximately 50% of the T-cell preference. Three nucleotide substitutions located between the enhancer and the consensus CCAAT box have been exchanged between FeLV-myc and GA LTR and are currently being evaluated. To date, the data obtained clearly show that both duplications and nucleotide substitution play a critical role in determining cell type preference.

Publications:

Braun, M. J., Deininger, P. L. and Casey, J. W.: Nucleotide sequence of a transduced myc gene from a defective feline leukemia provirus. J. Virol. 55: 177-183, 1985.

Patents:

None

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01CP05389-03 LVC

PERIOD COVERED

October 1, 1985 to September 30, 1986

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Development of Reproductive-Endocrine-Genetic Strategies in Animal Species

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	Stephen J. O'Brien	Chief	LVC	NCI
Others:	David E. Wildt	Guest Researcher	LVC	NCI
	Cheryl A. Winkler	Biologist	LVC	NCI
	Janice S. Martenson	Microbiologist	LVC	NCI

COOPERATING UNITS (if any)

Department of Animal Health, National Zoological Park, Washington, DC (M. Bush);
 Veterinary Resources Branch, DRS, NIH, Bethesda, MD (P. M. Schmidt, K. L.
 Goodrowe, M. C. Schiewe, J. G. Howard)

LAB/BRANCH

Laboratory of Viral Carcinogenesis

SECTION

Genetics Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701-1013

TOTAL MAN-YEARS:

1.3

PROFESSIONAL:

1.1

OTHER:

0.2

CHECK APPROPRIATE BOX(ES)

- (a) Human subjects (b) Human tissues (c) Neither
 (a1) Minors
 (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The objective of this project is to increase the reproductive-endocrine-genetic data base of nondomesticated, wildlife species. Program approaches emphasize investigation of domestic animals and basic reproductive-endocrine-genetic factors which appear as the most critical prerequisites to the application of artificial breeding strategies. A multidisciplinary approach targeted toward female and male reproduction and genetics is employed. The use of domestic animal models permits testing the efficacy of research concepts before adaptation to rarer, nondomestic species. Areas of effort in the female include: (1) hormonal evaluation for correlation of endocrine profiles of pituitary-ovarian function and as an index of the influence of manipulative stress, (2) ovulation induction through the administration of exogenous gonadotropins to optimize the timing of the ovulatory event, and (3) in vitro fertilization and embryo collection, culture, freezing and transfer as techniques for cryobanking genetic stock and for improving reproductive potential. These methodologies are being applied to the development of microinjection of molecularly cloned genes which participate in transformation and inborn errors. Emphasis has been applied to the collection, in vitro culture, freezing, and micromanipulation of embryos of mouse, cat and miniature swine, animal models for both rare species and the study of human disease. Areas of effort in the male include: (1) semen collection and evaluation to characterize ejaculate norms and correlate these findings to the level of genetic polymorphism in wildlife populations (see Z01CP05367-02 LVC), (2) semen handling and cryopreservation to increase spermatozoal viability and to establish optimal methods for chronic storage of genetic material, and (3) hormonal evaluation to improve the understanding of pituitary-gonadal-adrenal relationships with particular emphasis on the marked differences in stress responses among taxonomically related wildlife species. Together, these generalized research concepts permit rapid expansion of physiological-genetic norms for rare species and also improve methods of assessing fertility potential or genetic status to optimize management efforts for propagation.

PROJECT DESCRIPTIONNames, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Stephen J. O'Brien	Chief	LVC	NCI
David E. Wildt	Guest Researcher	LVC	NCI
Cheryl A. Winkler	Biologist	LVC	NCI
Janice S. Martenson	Microbiologist	LVC	NCI

Objectives:

To increase and integrate the reproductive-endocrine-genetic data base of domestic and nondomestic species, thereby permitting improved propagation and embryological manipulation through natural or artificial breeding or altered approaches to captive animal management.

Methods Employed:

The following techniques were employed: (1) radioimmunoassay for analyses of hormone concentrations in blood, (2) surgical laparoscopy for direct examination of ovarian activity and recovery of oocytes, (3) hormonal induction of ovulation and sexual behavior using gonadotropic therapy, (4) surgical procedures for embryo recovery and transfer, (5) programmable freezing for cryopreserving embryos, (6) electroejaculation for semen collection, (7) light and electron microscopy for evaluation of spermatozoal viability and structural integrity, (8) dry ice-pelleting procedures for spermatozoal freezing, (9) *in vitro* establishment of primary cultures from embryos and skin biopsies, and (10) biochemical genetic procedures for determining extent and character of genetic variation in populations and for monitoring of paternity and maternity exclusion in embryo transfers.

Major Findings:

1. Estrous synchronization and hormonal induction of ovulation. A prerequisite to successful artificial insemination or embryo transfer is the effective induction of estrous and accurate timing of ovulation. Hormonally controlling estrus and stimulating ovulation frequently results in highly variable responses in domestic species. Consequently, considerable variation also is expected after similar treatment of nondomestic animals. Data collected from domestic species continues to be extrapolated for developing hormonal treatments for wildlife species. Our early studies demonstrate the efficacy of inducing estrus behavior and ovarian activity in domestic cats with serial injections of follicle stimulating hormone (FSH-P). Additionally, electroejaculation was determined to be an effective method for collecting semen from male domestic cats and artificial insemination of hormonally treated females resulted in the births of live offspring. These results provided incentive and a biological basis for similar artificial breeding attempts in the cheetah, an endangered species. Of 30 mature female cheetahs treated with serial injections of FSH-P, 23 (76.7%) responded by ovulating, on the average 5.6 corpora lutea/animal. Ovulating cheetahs were artificially inseminated; however, only one female displayed behavioral evidence

of sexual receptivity and none of the females became pregnant. The negative pregnancy data were important in illustrating the need for more basic research and improved biological sources of exogenous gonadotropin. As a result, more detailed studies were conducted in the domestic cat model. Of particular interest was the finding that repeated injections of FSH-P produced cystic-appearing ovarian follicles which frequently failed to ovulate even after ancillary (human chorionic gonadotropin or gonadotropin releasing hormone) hormonal therapy. Recent observations suggest that perturbations in endocrine function as a result of these anovulatory follicles were detrimental to fertilization and embryo quality. These data have stimulated interest in a related project concerning the in vivo bioassay of newly available pregnant mares' serum gonadotropin (PMSG) compounds. Because of a high molecular weight, PMSG has considerable therapeutic potential since a single injection often is sufficient to stimulate ovarian activity. Recently, bioengineered, ultrapurified PMSG compounds have been acquired and are being tested in the mouse and cat system. The preliminary data suggest that these PMSG compounds will be routinely effective for stimulating follicular maturation for the ongoing in vitro fertilization program.

Comparative ovulation induction studies also continued in domestic and nondomestic hoofstock. Concepts developed and expanded in domestic sheep and cattle were effectively applied to a nondomestic bovid, the scimitar-horned oryx. The oryx corpus luteum lysed in response to a domestic cow dose prostaglandin F2 α . Administering FSH-P or PMSG produced highly variable results in the oryx. Certain females were relatively refractory to the conventional superovulatory therapies; however, occasional multiple ovulatory responses (11-16 corpora lutea/female) were achieved allowing recovery of embryos from this rare species.

2. In vitro fertilization, embryo collection, culture, freezing and transfer. Embryo techniques, similar to those extensively used in domestic farm stock, could play a valuable role in propagating selected rare species. Of particular importance is the potential of freeze-preserving embryos, thereby assuring maintenance of rare gene pools or providing a means of incorporating new genetic material into captive populations with limited genotypic diversity.

Comprehensive basic and applied embryo studies continued in a number of domestic animal models. A major collaborative effort continued with the Veterinary Resources Branch of the NIH in continuing an embryo cryopreservation bank for numerous genotypes of inbred and outbred laboratory mice. In 1985, more than 36,700 mouse embryos were collected, 8,000 of these embryos being permanently banked as an invaluable resource and the remainder used in comparative biomedical studies. To date, a minimum of 1000 embryos have been permanently stored from ten strains (inbred) and three stocks (outbred) from the Veterinary Resources Branch's mouse genetic resource. Major findings in the past year continued to emphasize the role of genotype on the ability of the embryo to survive a cryostress. Specifically, the results indicated that the proportion of inbred (purebred) embryos surviving freezing varied significantly among stocks and strains. Furthermore, embryo viability post-freezing was affected by mating an inbred male to a genotypically different female, suggesting a genetic influence of them on the ability of embryos to survive cryopreservation. Together the data suggested that the efficiency of a mouse embryo banking program was significantly influenced by and dependent upon genotype within the species.

Related studies focused on the influence of selected environmental factors on in vitro embryo culture. Ethylene oxide (Eto) gas-sterilization has been considered the most effective means of sterilizing heat-labile materials used in embryo transfer and embryo culture systems. Mouse embryos were cultured in vitro in polystyrene (plastic) dishes to analyze the toxicity potential of absorbed-retained Eto. Aeration of cultureware for 24 to 36 hours resulted in suboptimal embryo development demonstrating that Eto sterilization of polystyrene culture dishes required careful attention to aeration duration to avoid potentially toxic effects on embryo development.

A major embryological emphasis continued in the domestic cat. A second litter of kittens was born as a result of embryo transfer. However, the unreliability of the ovulation induction treatment and the need for early-staged embryos stimulated a new course of research. Recovery of high quality embryos and the pregnancy rates after transfer appeared to be compromised by the use of exogenous gonadotropins in both donor and recipient cats. A potential alternative is in vitro fertilization (IVF) which involves the extracorporeal fusion of mature oocytes and spermatozoa. The IVF approach has wide potential application in both domestic and rare, wild felids and could be an invaluable method for producing pronuclear stage embryos for gene injection studies. To have routine application, oocytes must be recovered without traumatizing the oviduct which is predisposed to adhesion formation. This obstacle has been circumvented by aspirating follicular oocytes using transabdominal laparoscopy. Using a purified source of PMSG, gonadotropin hormonally treated queens were subjected to laparoscopic oocyte aspiration. Washing oocytes were incubated with electroejaculated spermatozoa. Approximately 90% of cell punctured follicular resulted in recovered oocytes. Based on observations of pronuclear formation or blastomere formation and cleavage, approximately 34% of cell-cultured ova were judged fertilized. Embryos have advanced to the morula stage in culture.

A second strategy for obtaining early stage feline embryos is exogenous fertilization, the fusion of homologous oocytes and spermatozoa in a heterologous host species. Preliminary studies were initiated in which domestic cat oocytes and spermatozoa were incubated together for 24 hours in the rabbit oviduct. Recent results determined that early cleavage and blastomere formation occurred, thus obviating the absolute need for sophisticated culture systems required for conventional IVF.

3. Hormonal evaluations and the influence of stress. Specific radioimmunologic analyses of blood sera can be used to determine circulating concentrations of numerous protein and steroid hormones. Hormonal information allows the plotting of endocrine profiles which serve as indices of normal or abnormal pituitary-gonadal-adrenal function. These data provide information on duration of reproductive cycles and the influence of environmental factors, including stress, on reproductive-endocrine function. Because many species of wildlife are presumed highly susceptible to stress, it is critical that manipulations imposed on animals (anesthesia, electroejaculation, laparoscopy and laparotomy) be evaluated for acute and chronic effects on general animal health, including reproductive performance.

In 1985, studies emphasized the relationship of adrenal function to pituitary-gonadal activity in captive and free-ranging felids. A circannual analyses of

the clouded leopard indicated that this species, compared to other felids, produced extraordinarily high circulating concentrations of cortisol, suggesting stereotypic differences in adrenal function among taxonomically related Felidae. Furthermore, cortisol levels in clouded leopards subjected to as few as one immobilization/month responded with a chronic increase in adrenal function. Of significance was the finding that gradually increased adrenal activity had no effect over time on luteinizing hormone or testosterone secretion or the ability of males to produce spermic ejaculates throughout the year.

Related efforts focused on surveying adrenal-pituitary-gonadal activity in captive versus free-ranging cheetahs. Reproduction performance of the cheetah in captivity is notoriously poor with the male producing, on the average, 70% morphologically abnormal spermatozoa/ejaculate. Previous studies determined that the cheetah was genetically monomorphic, a possible explanation for compromised male reproductive performance. A second possibility was that increased adrenal function attributable to captivity stress adversely affected ejaculate quality, potentially through disruption in gonadotropin or gonadal steroid production. Adrenal/gonadal endocrine activity was similar between captive and free-ranging cheetahs indicating that spermatozoal diversity originated not because of environment but as a result of the extreme genetic monomorphism observed universally in the species.

4. Spermatozoal evaluation, freeze-preservation and handling. Basic and applied semen studies continued to emphasize establishing ejaculate norms for many nondomestic species, both captive zoo animals and those free-ranging in the native habitats. Considerable efforts were made to expand observations made that certain wild species of Felidae produce ejaculates with high proportions of abnormal sperm cells. In particular, a year long fertility analyses was made in the captive clouded leopard. The results established and interrelated electroejaculate-endocrine norms for the species and demonstrated that recoverable motile spermatozoa could be obtained throughout the year. The clouded leopard consistently produced high proportions of pleiomorphic spermatozoa; other related data suggest this to be the result of either a hyperadrenal function or a relatively compromised genotype. In a related study, ejaculate characteristics were measured in 20 captive cheetahs in North American zoos and 8 free-ranging males in eastern Africa (Tanzania). Using a standardized semen collection procedure, numbers of motile spermatozoa were comparable between groups. Of the spermatozoa collected per electroejaculate, between 70% and 76% were morphologically abnormal in the two populations. The similarity between groups confirmed that the unique seminal characteristics of the cheetah were unrelated to environment but likely dependent on the genetic uniformity of the species.

Strategies also continued for establishing optimal methods for cryobanking spermatozoa. This concept was developed primarily with the realization that marked differences exist in post-thaw sperm survival when comparing various cryoprotectants. In the past year, detailed studies were completed involving the freeze-storage of spermatozoa collected from free-ranging African elephants. Ejaculates were aliquoted in each of seven different cryoprotective diluents, cooled and frozen in pellet form or in straw containers. Post-thaw spermatozoal motility, motility duration *in vitro* and acrosomal integrity were greatest using a Beltsville Farm 5+ fructose diluent. Although the cooling-equilibration

interval had no effect, a more rapid initial cooling rate enhanced post-thaw acrosomal integrity. The thawing media tested exerted a negligible influence; however, *in vitro* viability was greatly prolonged by maintaining thawed ejaculates at 21°C rather than 37°C.

Publications:

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- Schiewe, M. C., Howard, J. G., Stuart, L. S., Goodrowe, K. L. and Wildt, D. E.: Human menopausal gonadotropin (hMG) for superovulation of sheep. In Shille, V. M. (Ed.): International Embryo Transfer Society and Theriogenology. Los Altos, Geron-X, Inc., 1985, Vol. 23, p. 227.
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- Schiewe, M. C., Schmidt, P. M., Rall, W. F. and Wildt, D. E.: Influence of cryoprotectant and plunge temperature on post-thaw viability of bovine embryos. In Ewing, L. L. (Ed.): Proceedings Society for the Study of Reproduction and Biology Reproduction. Champaign, Society for the Study of Reproduction and Biology Reproduction, 1985, Suppl. 1, Vol. 32, p. 98.

Schiewe, M. C., Schmidt, P. M. and Wildt, D. E.: Toxicity potential of ethylene oxide residues in straw containers used for embryo cryopreservation. In Shille, V. M. (Ed.): Proceedings International Congress on Animal Reproduction and Artificial Insemination. Champaign, University of Illinois, 1986, Vol. 25, p. 194.

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Wildt, D. E.: Reproductive techniques of potential use in the artificial propagation of nonhuman primates. In Heltne, P. G. (Ed.): The Lion-Tailed Macaque: Status and Conservation. New York, Alan R. Liss, 1985, pp. 161-194.

Wildt, D. E., Howard, J. G. and Bush, M.: Ejaculate characteristics and adrenal-pituitary-gonadal relationships in clouded leopards evaluated throughout the year. In Fowler, M. (Ed.): Proceedings American Association of Zoo Veterinarians. Davis, American Association of Zoo Veterinarians, 1985, Vol. 200, pp. 33-34.

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Patents:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01CP05401-02 LVC
PERIOD COVERED October 1, 1985 to September 30, 1986		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Molecular Basics of Retroviral Transcriptional Transactivation		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
PI:	James W. Casey	Senior Staff Fellow LVC NCI
Other:	David D. Derse	Guest Researcher LVC NCI
COOPERATING UNITS (if any) None		
LAB/BRANCH Laboratory of Viral Carcinogenesis		
SECTION Genetics Section		
INSTITUTE AND LOCATION NCI, NIH, Frederick, Maryland 21701-1013		
TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.0	0.5	0.5
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) The bovine leukemia virus (BLV) is related only to the HTLV-I and -II retroviruses in genetic structure and DNA sequence. A property which further emphasizes the relationship between these retroviruses is that they contain additional open reading frames (ORFs) located 3' to the envelope gene, which potentially encode transcriptional and immortalizing functions. One of these, the long open reading frame (LOR), has been molecularly reconstructed from a cloned provirus into an expression vector containing differentially regulated promoter units. Co-transfection of the BLV LOR with the BLV long terminal repeat (LTR) establishes that BLV LOR can, in itself, confer high levels of transcriptional activity on the BLV LTR. The human retrovirus HTLV-I has been shown to immortalize T-cells <u>in vitro</u> ; however, attempts to immortalize bovine or human blood lymphocytes with BLV have proven negative. We have taken an alternative approach to identify a potential BLV-encoded transforming function by investigating the status of the BLV provirus in cell lines established from BLV-induced B-cell lymphosarcomas. One cell line, NBC-13, has amplified a BLV deletion type provirus approximately 20-fold over single copy genes. The BLV deletion type provirus has been molecularly cloned and sequenced. Results from these analyses show that a protease fusion to the 3' pre-px gene region has occurred and that deletion type proviruses most probably arise from reintegration of spliced BLV cDNA transcripts. The function of this potential BLV gene product with regards to immortalizing properties is currently being assessed.		

PROJECT DESCRIPTIONNames, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

James W. Casey	Senior Staff Fellow	LVC	NCI
David D. Derse	Guest Researcher	LVC	NCI

Objectives:

Bovine leukemia virus (BLV), like HTLV-I, contains an additional segment of DNA, 3' to the envelope gene, that has the potential to encode 3 proteins. We have determined the function of the longest open reading frame (LOR) and find that it will trans-activate a small set of target sequences in the BLV LTR. Since BLV induces B-cell lymphocytosis in vivo, an additional viral function which immortalizes B-cells is postulated. Our objective is to determine the function of all open reading frames in the provirus and relate these functions to the pathogenesis of the virus.

Methods Employed:

The following techniques were employed: (1) genomic molecular cloning in lambda vectors, (2) DNA-mediated transfection, (3) SV40 chloramphenicol acetyl transferase (CAT) assays to measure promoter/enhancer activity, (4) DNA sequence analysis using the Sanger shotgun technique, and (5) Southern blotting and mapping of eukaryotic genes.

Major Findings:1. Bovine leukemia virus encodes a transacting factor.

The expression of BLV is highly restricted in nature and its LTR was shown to be a functional promoter only in BLV-producing cell lines, suggesting that virus gene products might positively regulate expression. Based upon similar studies with HTLV-I, a candidate gene with trans-acting function would be the long open reading frame located in the post envelope region of the virus. A first assessment of function of this viral region was made by placing the entire envelope and LOR under the control of either the SV40 or RSV promoter. The logic in using this construct lies in the observation that the long open-reading frame requires a pre-envelope splice which places an initiation methionine codon next to LOR. Co-transfection of this construct with the BLV-LTR-CAT construct shows that LOR can trans-activate the LTR. Sequences between the splice donor (pre-envelope) and splice acceptor (post-envelope) have been deleted in the LOR construct without affecting the trans-acting function. A possibility still exists that another protein could be encoded in a second reading frame. This would utilize a second initiator methionine which is present in the LOR construct. Experiments have been initiated to remove the second initiation codon and results from this study will allow an unambiguous assignment of LOR function. The precise target in the BLV-LTR for LOR trans-activation has been determined. We had previously shown that an element located 80-150 base pairs (bp) upstream of the TATA box was necessary to observe activation. However, the BLV LTR remains responsive to LOR activation

even when this sequence is deleted. Further deletion experiments show that sequences 20-70 bp 5' of the TATA box are essential for LOR activation. Thus, the BLV LTR contain at least two essential elements within the LTR that respond to trans-activation. The presence of two LOR responsive elements and sequences downstream of the CAP site, which we previously demonstrated to augment gene expression, suggest several potential levels of control of BLV expression.

2. Isolation of an amplified deletion type BLV provirus from a lymphosarcoma cell line.

The molecular basis for BLV or HTLV-I-induced neoplasms remain obscure. HTLV-I will immortalize T-cell in vitro, while no such activity is found in BLV. Since BLV displays a highly restricted host range in vitro, it is difficult to directly compare these two viruses or to assume that BLV is nonimmortalizing. Conversely, BLV will induce a persistent B-cell lymphocytosis in vivo, suggesting that this virus does contain an immortalizing function. We have characterized four BLV infected cell lines, established from bovine B-cell lymphosarcomas, for the presence, structure, and location of proviruses. All cell lines were monoclonal with respect to cellular integration sites. Further, all cell lines encoded trans-acting factors as assayed by co-transfection with the BLV-LTR-CAT construct. However, one cell line, NBC-13, displayed a highly amplified BLV proviral copy number (approximately 20 per cell). The striking feature of the amplified proviruses was the observation that all had suffered a 5 kilobase deletion spanning polymerase and envelope. To obtain the detailed structure and sequence of these deletion type proviruses, we molecularly cloned 12 of these proviruses. These proviruses have not transduced cellular genes and represent pure deletions. Four proviruses have been mapped and data indicate that they are identical in size and that the deletion has occurred at the same location in each. One deletion provirus has been sequenced through the junction of the deletion. The result of this analysis shows that the C-terminus of protease has been deleted and the remaining truncated gene fused to the pre-px region of the genome. A prediction from the DNA sequencing data is that a new protease fusion gene would be synthesized. Further, a consensus splice donor appears to have spliced to a putative splice acceptor at this junction generating the protease fusion gene. The implications are that the deletion type proviruses are the results of a BLV transcript being reverse transcribed and reintegrated. The observation that these proviruses are amplified suggest that they play a role in maintaining the transformed phenotype and that the protease fusion gene product may be an essential gene product in this process.

Publications:

Derse, D. D. and Casey, J. W.: Two elements in the bovine leukemia virus long terminal repeat that regulate gene expression. Science 231: 1437-1440, 1986.

Patents:

None

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01CP05404-02 LVC

PERIOD COVERED

October 1, 1985 to September 30, 1986

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Growth Modulation of raf Associated Tumors

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	Ulf R. Rapp	Chief, Viral Pathology Section	LVC	NCI
Others:	Stephen Storm	Guest Researcher	LVC	NCI
	John L. Cleveland	Staff Fellow	LVC	NCI
	Gunamani Sithanandam	Guest Researcher	LVC	NCI
	John D. Minna	Senior Investigator	COP	NCI

COOPERATING UNITS (if any)

Litton Bionetics, Inc., Frederick, MD (S. Sukumar)

LAB/BRANCH

Laboratory of Viral Carcinogenesis

SECTION

Viral Pathology Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701-1013

TOTAL MAN-YEARS:

2.3

PROFESSIONAL:

1.5

OTHER:

0.8

CHECK APPROPRIATE BOX(ES)

- (a) Human subjects (b) Human tissues (c) Neither
- (a1) Minors
- (a2) Interviews

SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

The identification of the v-raf-1 oncogene from the defective retrovirus 3611-MSV has allowed the isolation of a new family of proto-oncogenes. The active homolog of v-raf, c-raf-1, is located at position p25 on chromosome 3 in man, a site that is frequently altered in a variety of neoplasia, including small cell lung carcinoma. Moreover, transforming versions of c-raf-1 have been identified in human stomach cancer and glioblastoma. Expression of c-raf-1 mRNA and protein in human lung carcinoma cell lines occurs at levels that are in excess of those found in NIH 3T3 cells transformed by c-raf MSV. To study the role of raf in lung carcinogenesis *in vivo*, we have developed an animal model where 90% of newborn mice treated transplacentally with ethylnitrosourea and promoted at birth with butylated hydroxytoluene develop lung tumors and lymphomas within 5 to 14 weeks. High c-raf-1 expression in tumors and established cell lines has been determined by Northern blot and immunoblotting analyses. DNA prepared from both lung tumors and lymphomas is transforming for NIH 3T3 cells as determined by DNA-transfection. The nature of the activated transforming DNA is currently being examined. Moreover, we have now defined conditions using growth modulators and hormones for the differential inhibition of raf-transformed, but not untransformed, control cell lines *in vitro*. These regimens are now being tested in our animal model system, in conjunction with vaccination experiments using purified raf protein, to examine their potential inhibitory actions on the development of these tumors.

PROJECT DESCRIPTIONNames, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Ulf R. Rapp	Chief, Viral Pathology Section	LVC	NCI
Stephen Storm	Guest Researcher	LVC	NCI
John L. Cleveland	Staff Fellow	LVC	NCI
Gunamani Sithanandam	Guest Researcher	LVC	NCI
John D. Minna	Senior Investigator	COP	NCI

Objectives:

To develop an animal model for induction of lung carcinoma and define regimens for prevention and/or reversal of neoplasms involving raf oncogenes. In order to increase the incidence of tumor induction, NFS (female) mated with AKR (male) were used for transplacental inoculation.

Methods Employed:

Human lung tumor cell lines of all histological types were examined for expression of raf oncogene RNA (Northern) and protein (immunohistochemistry, immunofluorescence, Western blot, protein-kinase assay).

For induction of lymphomas and lung adenocarcinoma in mice, pregnant females were injected transplacentally with ethylnitrosourea (ENU). To accelerate tumor development in the offspring, weanling age F1 mice were promoted with weekly injections of butylated hydroxytoluene (BHT). Vaccination of newborn mice with oncogene protein followed procedures previously developed for vaccination of high leukemia strains of mice with retroviral structural proteins.

Major Findings:

High level expression of normal size raf RNA and protein were found in 60% of all types of lung carcinoma. Comparison of the amount of raf protein in lung tumor versus 3611 MSV virus-transformed or LTR-c-raf-transformed cells showed it to be greater than fivefold excess in lung tumor cells. The c-raf-1 gene should, therefore, be considered as one of the genes involved in the development of human lung carcinoma.

Ninety percent of the animals treated with both initiator (ENU) and promoter (BHT) developed tumors within 5 to 14 weeks. The purpose of developing this rapid tumor induction model was to mimic development of tumor types that can also be induced by raf-transducing viruses so as to allow us to test for involvement of endogenous raf and other proto-oncogenes in the chemically induced transformation process. At the same time, we intend to employ reagents that show growth inhibitory effects specifically on v-raf transformed cells in vitro for possible modulation of tumor development in vivo.

Tumors induced by ENU and BHT contain transforming DNA as judged by transfection of NIH 3T3 cells. The identity of the transforming DNA is currently being examined.

One possible modulation that we considered was the animals' immune response to the product of oncogenes that may be involved in the development of tumor cells. We, therefore, vaccinated, treated and controlled mice with v-raf protein. In initial experiments with 150 mice, a fairly dramatic effect of this treatment was observed. In the group where animals had been treated with BHT, a doubling of the latency period for development of the earliest tumor was observed. Mice that had only been treated with ENU before vaccination showed an 80% increase in latency. This delay in the onset of tumor development upon vaccination was transient. A comparable incidence of lung adenocarcinoma and lymphoma was observed in treated and control groups 20 weeks after treatment.

Multiple repeats of the experiments have shown that a delay in tumor induction by raf protein vaccination was only apparent during the early phase of tumor development, between 5 and 13 weeks.

Publications:

Rapp, U. R., Storm, S. and Cleveland, J. L.: Oncogenes: Clinical relevance in hematology and blood transfusion 31. In Neth, R., Gallo, R., Greaves, M. and Janka, K. (Eds.): Modern Trends in Human Leukemia VIII. Stuttgart, Wissenschaftliche Verlagsgesellschaft mbH (In Press)

Patents:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER
PERIOD COVERED October 1, 1985 to September 30, 1986		Z01CP05414-03 LVC
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Isolation and Molecular Characterization of Human and Primate Retroviruses		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
PI:	Raoul E. Benveniste	Medical Officer LVC NCI
Others:	James W. Casey	Senior Staff Fellow LVC NCI
	Gisela Fanning-Heidecker	Visiting Fellow LVC NCI
COOPERATING UNITS (if any) Univ. Washington Primate Res. Center, Seattle, WA (E. Clark, W. Morton, H. Ochs, M. Thouless, C. Tsai); LBI, Frederick, MD (M. Gonda, L. Henderson, S. Oroszlan); PRI, Frederick, MD (L. Arthur); Fairfax Hospital, Fairfax, VA (L. Eron, D. Poretz)		
LAB/BRANCH Laboratory of Viral Carcinogenesis		
SECTION Viral Leukemia and Lymphoma Section		
INSTITUTE AND LOCATION NCI, NIH, Frederick, Maryland 21701-1013		
TOTAL MAN-YEARS: 1.2	PROFESSIONAL: 0.7	OTHER: 0.5
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input checked="" type="checkbox"/> (b) Human tissues <input type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p>At the University of Washington Primate Research Center (WPRC), several macaque species show an acquired immunodeficiency syndrome (simian AIDS, SAIDS) characterized by lymphocytopenia, opportunistic infections, and a retroperitoneal fibromatosis tumor (RF). Numerous type D retroviruses, designated SAIDS-D/W, have been isolated by cocultivation of tissues and blood with lymphocyte and monolayer cultures. These SAIDS-D/W isolates can be distinguished from all other type D retroviruses by antigenicity and restriction enzyme pattern.</p> <p>Another retrovirus has been isolated on the HuT 78 cell line after cocultivation of a lymph node from a <u>Macaca nemestrina</u> that had died with lymphoma in 1982 at the WPRC. This isolate, designated MnIV (WPRC-1) (<u>M. nemestrina</u> immunodeficiency virus), shows the characteristic morphology of a lentivirus and replicates to high titers in various human and primate lymphocyte lines. The relatedness of MnIV to other lentiviruses (HTLV-III/LAV, EIAV, visna) was examined immunologically and by N-terminal amino acid sequence analysis of the major viral <u>gag</u> protein. Fourteen of the 24 N-terminal residues of MnIV p28 and HTLV-III/LAV p24 are identical. These results indicate that MnIV belongs to the same lentivirus family as HTLV-III/LAV, but is only partially related to these human AIDS retroviruses. SAIDS-D/W and MnIV have been inoculated into several primate species; the former virus causes RF tumors and the latter results in severe immunosuppression.</p> <p>A retrovirus isolated from an AIDS patient and designated human immunodeficiency virus, Frederick isolate 3 (HIV[FRE-3]) has a defect in processing of the <u>gag</u> precursor protein and produces immature virus particles. The virus is being biologically cloned on sheep choroid plexus cells and will be molecularly cloned in order to study the biochemical basis of the defect.</p>		

PROJECT DESCRIPTIONNames, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Raoul E. Benveniste	Medical Officer	LVC	NCI
James W. Casey	Senior Staff Fellow	LVC	NCI
Gisela Fanning-Heidecker	Visiting Fellow	LVC	NCI

Objectives:

To identify and characterize the causative agent of simian acquired immune deficiency syndrome (SAIDS) and to propagate the virus to high titers in various cell lines. To examine, by molecular hybridization techniques, the homology between this virus and other primate retroviruses. Biological and molecular clones and a restriction map of the isolate will be obtained. The viral proteins will be purified and antisera to the individual proteins will be raised in rabbits in order to develop specific immunological assays.

To examine the prevalence of the SAIDS viral isolate and of any other lymphotropic viruses in the University of Washington primate colony and to correlate clinical status with viremia and the presence of viral antibodies. To determine whether the SAIDS-D isolate, including biological and molecular clones, can cause the disease when inoculated into macaques. To prevent SAIDS by vaccination of animals with disrupted virus or purified viral proteins. To characterize lymphotropic lentiviruses isolated from various primate species for their extent of antigenic and molecular similarity to various human AIDS viral isolates. To determine whether these lentivirus isolates can cause disease in primate species. To determine the prevalence of these various primate virus isolates in feral populations. To determine the molecular basis for the lack of infectivity of some human AIDS isolates.

Methods Employed:

Virus isolation was attempted using cell lines that have previously been employed for the isolation of other primate and human retroviruses. Fresh tumors, whole blood, or sera obtained from macaques were cocultivated with various cells and the supernatant assayed weekly for reverse transcriptase activity. Cloned retroviral DNA will be used as a probe for detection of related DNA sequences in primates. Viral proteins were purified by high pressure liquid chromatography (HPLC) and SDS-gels. Antigens and antibodies to the purified SAIDS-D and MnIV viral proteins were detected by radioimmunoassays and by Western immunoblot techniques.

Major Findings:

1. Isolation and molecular characterization of type D retroviruses from macaques with SAIDS and retroperitoneal fibromatosis (RF).

Since the original isolation of a type-D retrovirus (SAIDS-D/W) from the RF tissue of an immunodeficient rhesus monkey, a total of 40 additional isolates have been obtained from various species of macaques at the University of Washington Primate

Center. Twenty-one of these isolates were from RF tissues obtained from four species of macaques (M. nemestrina, M. fascicularis, M. mulatta, M. fuscata); the additional 19 isolates were obtained from macaques with recurrent diarrhea, significant weight loss, or lymphadenopathy. No viruses have been isolated from healthy macaques or from the baboon colony at the same primate center. The SAIDS-D/W viral gag proteins have been purified by HPLC and the N-terminal region sequenced in collaboration with Drs. L. Henderson and S. Oroszlan. The proteins purified include p4, p10, p12, p14, p27, and a p18 phosphoprotein. Competition radioimmunoassays (RIA) performed with these proteins reveal that the type D isolates from the various primate centers (Oregon, California, and New England) can be distinguished from SAIDS-D/W by using the p4 and p10 RIA.

2. Isolation and characterization of lentiviruses from primates.

A retrovirus has been isolated on the human T-cell line, HuT 78, after cocultivation of a lymph node from a pig-tailed macaque (M. nemestrina) that had died with malignant lymphoma in 1982 at the University of Washington Primate Research Center. This isolate, designed MnIV (WPRC-1) (M. nemestrina immunodeficiency virus, Washington Primate Research Center isolate) shows the characteristic morphology of a lentivirus and replicates to high titers in various lymphocyte lines of human and primate origin. SDS-PAGE of purified MnIV reveals multiple bands of structural proteins, including a major viral gag protein of 28 kilodaltons, that do not comigrate with the viral proteins of a human immunodeficiency virus (HIV[FRE-1]) also isolated on HuT 78 cells. The relatedness of MnIV to other lentiviruses (HTLV-III/LAV, EIAV, visna) was examined immunologically and by N-terminal sequence analysis of the viral p28 gag protein. The immunoassays reveal cross-reactivity only between MnIV p28 and HTLV-III/LAV p24 and sequence analysis shows that 14 of the 24 N-terminal residues of MnIV p28 and HTLV-III/LAV p24 are identical. These results indicate that MnIV belongs to the same lentivirus family as HTLV-III/LAV, but is only partially related to these human retroviruses. MnIV viral preparations contain no detectable 70S viral RNA; the virus is, therefore, being cloned with a 27 base oligomer from a 9 amino acid region in the N-terminal portion of the major gag protein that is identical in MnIV and HTLV-III.

3. Seroepidemiology of primate type D viruses (SAIDS-D/W) and primate lentiviruses (MnIV).

The primate colony at the University of Washington Primate Research Center (WPRC) consists of 1300 macaques, primarily M. nemestrina. A total of 224 animals were screened for antibodies to SAIDS-D/W, MnIV, HTLV-I and HTLV-III by RIA for the major core antigens, and by ELISA and Western immunoblot techniques. None of the animals had antibodies to MnIV or HTLV-III, and 1% had antibodies to HTLV-I. In marked contrast, 45% of the animals under one year of age had antibodies to the type D retrovirus; by age three, 98% of the animals tested were antibody positive. Several species of feral primates are also being examined for naturally occurring antibodies.

4. Inoculation of macaques with SAIDS-D/W and MnIV.

In order to establish whether the SAIDS-D viral isolate is the etiologic agent of SAIDS, 19 animals have been inoculated with biologically cloned SAIDS-D/W virus

(10^6 virus particles) in two separate studies. Five animals have become viremic; one of these has died with symptoms of SAIDS and the other four have developed histologically confirmed RF. These five animals are SAIDS-D antibody negative. An additional ten animals have developed high titers of neutralizing antibodies to SAIDS-D; all are presently healthy. Naturally occurring and experimentally induced antibodies to the SAIDS-D/W virus neutralized SAIDS-D/W virus in vitro but not other type-D virus, such as the langur endogenous virus or Mason-Pfizer monkey virus. Macaques will be inoculated with a vaccinia-SAIDS-D/W recombinant (in collaboration with Dr. Shiulok Hu) in order to test if antibodies to SAIDS-D/W envelope glycoproteins are an effective antiviral vaccine. Three M. mulatta, three M. nemestrina, and two baboons (P. cynocephalus) have also been inoculated intravenously with end-point diluted MnIV grown in HuT 78 cells (10^3 infectious virus particles). MnIV could be isolated from the lymphocytes of all six macaques; five of the macaques developed neutralizing antibody titers and are healthy 20 weeks into the study. The sixth animal did not develop an antibody response and died at 14 weeks after exhibiting weight loss and an acute drop in $T4^+$ helper lymphocytes. Both baboons have remained virus and antibody negative.

5. Isolation, biological cloning, and characterization of an AIDS virus (HIV[FRE-3]) with a defect in processing of gag viral proteins.

A retrovirus isolated in our laboratory after cocultivation of the lymphocytes of a patient with AIDS with the HuT 78 cell line has been shown to accumulate large amounts of the gag precursor protein (Pr55). This isolate is designated human immunodeficiency virus, Frederick isolate #3 (HIV[FRE-3]). Electron microscopy (performed by M. Gonda) revealed that this isolate consisted of a mixed population of lymphocytes, some producing immature virus particles and some producing mature particles. This lymphocyte culture is being cloned in a sheep choroid plexus cell line in order to isolate a pure population of lymphocyte-producing defective virus particles. The virus will be molecularly cloned to study the basis of the defect.

6. Antibodies to HTLV-III/LAV in patients receiving immunoglobulin.

Immunoglobulin produced from large pools of plasma has been used successfully to protect susceptible patients from infections by providing passive immunity. We have tested 63 sera obtained from 23 patients with primary immunodeficiency syndromes between 1980 and 1986 before and immediately after immunoglobulin infusion. Using Western immunoblots, we found 52 sera to be HTLV-III/LAV antibody positive. There was a clear correlation between the amount of antibody present after infusion and the titer of antibody in the immunoglobulin lot administered. Since these patients cannot produce antibody, the data are consistent with an exogenous origin of the antibody and suggest that a positive titer for HTLV-III antibody in such patients is not the result of active infection with AIDS virus.

Publications:

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Patents:

None

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01CP05417-02 LVC

PERIOD COVERED

October 1, 1985 to September 30, 1986

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Molecular and Functional Characterization of raf Oncogenes

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

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Others:	Thomas W. Beck	Biotechnology Fellow	LVC	NCI
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1.0

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0.4

CHECK APPROPRIATE BOX(ES)

- (a) Human subjects (b) Human tissues (c) Neither
 (a1) Minors
 (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

We have been characterizing the molecular structure, expression and function of the raf oncogene family and examining the role they may play in the etiology of human neoplasia. Two functional genes, c-raf-1 and A-raf-1, related to v-raf (the active oncogene from 3611 MSV) have been identified. c-raf-1 is located on human chromosome 3p25, a site specifically altered in several human neoplasms including small cell lung carcinoma, familial renal carcinoma and mixed parotid gland tumors. The c-raf-1 gene is composed of 16 protein coding exons spanning more than 40 kb. It is expressed as a 3.4 kb and a 3.1 kb mRNA in human and murine cells, respectively, and is found in most cells and tissues examined. The c-raf protein is composed of 648 amino acids (73,000 daltons) and is located in the cytoplasm. A-raf is located on human chromosome Xp21-q11 near the locus for testicular feminization syndrome and Menkes syndrome. The A-raf-1 gene is expressed as a 2.6 kb mRNA in human and murine cells and shows a highly restricted tissue distribution of expression with highest levels attained in the epididymis of mice. The A-raf protein is composed of 606 amino acids (67,500 daltons) and its amino acid sequence shows 60% identity with c-raf. A role for raf family oncogenes in malignant transformation is indicated by the following evidence: (1) When incorporated into a retrovirus expression vector, truncated versions of c-raf and A-raf are transforming in vitro and in vivo. (2) c-raf-1 can be activated by promoter insertion in vitro. (3) Activated versions of raf family oncogenes have been identified in primary human stomach cancer, human glioblastoma and rat hepatocellular carcinoma. Functional assays of raf family oncogenes and their gene products have demonstrated that (1) amino terminal truncation of c-raf and A-raf protein enhances their serine/threonine kinase activity and their transformation potential, and (2) raf acts independent of ras in the signal transduction pathway of growth factors.

PROJECT DESCRIPTIONNames, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

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Objectives:

The specific objectives of these studies are (1) to define the mechanism(s) by which the raf family proto-oncogenes become activated and cause transformation in cells; (2) to molecularly and functionally characterize the members of the raf oncogene group and to determine how these genes are regulated in normal and transformed cells; (3) to define how raf oncogenes are associated with the initiation and maintenance of human cancers and to develop experimental regimens for control of raf-induced transformation; and (4) to identify and characterize genes and gene products which regulate raf expression and to determine the specific targets of "activated" and normal raf.

Methods Employed:

Standard recombinant DNA techniques were used to molecularly clone and subclone raf genomic and cDNA species from bacteriophage lambda gt10 libraries. raf clones were sequenced using dideoxy- and Maxam and Gilbert techniques and were computer analyzed. Specific cDNA clones were inserted into expression vectors and high levels of synthesis of raf polypeptides in E. coli demonstrated by protein gel and immunoblot analyses. raf-specific transcripts were identified by Northern blot analyses of poly(A)+RNA preparations from tumors or established cell lines. cDNA libraries from specific cell lines were made by standard techniques, cloned into lambda gt10, and specific raf cDNAs were isolated. Protein analyses of raf in normal and transformed cells were analyzed by metabolic labeling, immunoprecipitation and gel analyses, and raf-associated kinase activity was demonstrated using our established protocol.

Major Findings:

Since the isolation of the v-raf oncogene from an acutely transforming murine sarcoma virus (3611-MSV), significant progress has been made in the molecular and functional characterization of the raf oncogene family and the role they may play in the etiology of human neoplasia. The major findings are as follows:

1. The molecular cloning and sequencing of v-raf-related sequences in murine and human cells has identified two closely related active genes designated c-raf-1 and A-raf-1 belonging to the raf oncogene family. In addition, two processed pseudogenes have been identified: one related to c-raf-1 (designated c-raf-2) and one related to A-raf-1 (designated A-raf-2). All four raf-related loci have been mapped to specific chromosomal locations using somatic cell hybrids and in situ

hybridization to spreads of metaphase chromosomes. c-raf-1 has been localized to chromosome 3 at position p25, a site specifically altered in several human neoplasias including familial renal carcinoma, small cell lung carcinoma and mixed parotid gland tumors. In the murine genome, c-raf-1 maps to chromosome 6; restriction fragment length polymorphisms (RFLPs) have established that c-raf-1 maps approximately 16 centimorgans distal to the mouse immunoglobulin kappa light chain locus, which is altered in murine plasmocytomas. c-raf-2 has been localized to human chromosome 4 and using RFLPs, this gene has been found to be a clinically useful marker for Huntington's chorea. The A-raf-1 locus has been mapped in both mouse and human to the X chromosome. The human A-raf-1 locus has been regionally mapped to position p21 to q11 near the locus for testicular feminization (TFM) syndrome and Menkes syndrome which are linked in both mouse and man.

2. The functional human c-raf-1 gene is the most characterized raf family gene. The cloning of the c-raf-1 cDNA of 2.97 kb, which contains the complete coding sequence, has allowed the identification of all 16 coding exons of the gene spanning more than 40 kb. The mRNA size, as determined by Northern blotting experiments, is 3.4 and 3.1 kb in human and mouse, respectively, which is expressed in most cells and tissues examined, albeit at different levels. The cDNA encodes a protein of 648 amino acids. The cDNA was inserted into an E. coli expression vector and purified protein, as well as c-raf-1 synthetic peptides, were used to generate c-raf-1 specific antisera. These antisera were used for immunoprecipitations of metabolically labelled c-raf-1 protein and Western blotting analyses. From the data, we have identified a 74 kilodalton (kd) protein in murine and human cell lines. This is consistent with the predicted size based on the cDNA sequence (73 kd). Computer analysis of the complete amino acid sequence predicts that c-raf lacks extensive hydrophobic regions consistent with the results of cell fractionation and immunofluorescent experiments localizing the c-raf-1 protein to the cytoplasm of murine and human cells.

3. cDNAs corresponding to the A-raf-1 locus have been isolated from a murine spleen, human fetal liver and human T-cell libraries. The cDNA from the human T-cell library is 2.46 kb and contains a single open reading frame of 1818 nucleotides coding for a protein of 606 amino acids having a molecular weight of 67.5 kd. The predicted amino acid sequence shows 60% identity with the c-raf-1 and, taking into account conservative amino acid substitutions, the homology is greater than 85%. Northern blot analyses of mRNA from human and murine cells have identified a 2.6 kb mRNA. In contrast to c-raf-1, A-raf-1 shows a highly restricted tissue distribution of expression with the highest levels observed in the epididymis and intestine of mice. The cDNA was incorporated into an E. coli expression vector and the purified protein, as well as A-raf synthetic peptides, were used to generate A-raf antisera.

4. A role for the raf family oncogenes in neoplasia (both animal and human) seems likely not only from the chromosomal studies indicated above, but also from a variety of other experiments. First, truncated versions of c-raf-1 and A-raf-1 cDNAs, when incorporated into a retrovirus expression vector, cause transformation of cells in culture and induce fibrosarcomas and erythroid hyperplasias in newborn mice with a latency of 5 to 10 weeks when injected intraperitoneally. Secondly, transfection of mouse cells with the long terminal repeat of Moloney leukemia virus (Mo-LTR) led to the isolation of a malignant cell line containing an activated c-raf-1 locus in which the Mo-LTR was inserted into the fifth intron of

c-raf-1, thus resulting in the expression of high levels of a truncated version of c-raf-1 mRNA and protein via promoter insertion. Finally, activated versions of c-raf-1 (as judged by transfection of tumor cell DNA into NIH 3T3 cells and hybridization analyses of the transforming DNA) have been identified in a primary human stomach cancer, a human glioblastoma line and a rat hepatocellular carcinoma induced 2-amino 3-methylimidazo(4, 5-f) quinoline.

5. The function of the raf family oncogenes in normal and transformed neoplastic cells is unknown; however, some of their activities have become apparent. First, v-raf-1 and truncated versions of c-raf-1 and A-raf-1 have serine and threonine kinase activity as determined by an in vitro enzyme assay of immunoprecipitated raf protein. However, the full size (74 kd) c-raf-1 protein from normal cells is essentially inactive in this same assay. These results suggest a mechanism of oncogenic activation of raf family genes involving amino terminal truncation of the raf protein, thereby releasing the protein from negative regulation of its kinase domain. Secondly, a functional assay has been developed in which NIH 3T3 cells become growth arrested by microinjection of ras antibody and are then infected with oncogene-containing viruses and assayed for DNA synthesis as a measure for their ability to overcome the arrested growth. In this assay, A-raf MSV and 3611 MSV overcomes the antibody block, whereas other oncogenes (e.g., v-sis, v-fms and v-src) are unable to overcome the antibody block. Another functional assay utilizing flat revertants from Kirsten sarcoma virus-transformed cells (v-ras transformed) were found to be resistant to transformation by v-ras-containing viruses (Kirsten, Harvey and Balb MSV) and some viruses containing the oncogenes v-fes and v-src. However, we have found that these cells are susceptible to transformation by A-raf MSV and 3611 MSV. These results suggest that raf family oncogenes act independent of ras either through a signal transduction pathway not involving ras or one in which raf has a position downstream of ras.

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Beck, T. W., Gunnell, M., Kerby, S., Cleveland, J. L., Bonner, T. I. and Rapp, U. R.: The complete sequence of human A-raf, a new member of the raf family of oncogenes. Nucleic Acids Res. (In Press)

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Rapp, U. R., Cleveland, J. L. and Bonner, T. I.: The raf oncogene. In Gallo, R. C., Stehelin, D. and Varnier, O. E. (Eds.): Retroviruses and Human Pathology. Kansas City, The Humana Press, 1985, pp. 449-472.

Schultz, A. M., Oroszlan, S., Mueller, R., Moelling, K. and Rapp, U. R.: Identification and characterization of c-raf phosphoproteins in transformed murine cells. Mol. Cell. Biol. (In Press)

Patents:

None

NOTICE OF INTRAMURAL RESEARCH PROJECT

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October 1, 1985 to September 30, 1986

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Role of raf and myc Oncogene Synergism in Transformation

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

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0.7

OTHER:

0.1

CHECK APPROPRIATE BOX(ES)

- (a) Human subjects (b) Human tissues (c) Neither
- (a1) Minors
- (a2) Interviews

SUMMARY OF WORK (Use standard unabbreviated type. Do not exceed the space provided.)

We have examined the role of raf and myc family oncogenes in naturally occurring human tumors and in the mouse, and have determined their transforming properties and likely mechanisms of action. In the mouse, raf tumor induction was studied using a series of recombinant murine retroviruses. Viruses carrying only raf family oncogenes, including constructs with the cellular homolog of v-raf, c-raf-1, and a raf-related gene, A-raf-1, induce predominantly fibrosarcoma and erythroid hyperplasia. The J-2 virus, which contains both v-raf and v-myc, demonstrated a synergism of these two oncogenes in inducing hemopoietic and epithelial neoplasias in newborn mice with a greatly reduced latency period compared to disease induction by raf or myc viruses. Murine retroviruses containing only v-myc (J-3 and J-5) predominantly induce lymphomas (of both T and B lineage) but transform other hemopoietic and epithelial lineage cells as well, with a latency period which depends upon the replication efficiency of the pseudotyping MuLV. raf-induced neoplasias require specific interleukins (IL) for culturing in vitro, whereas cell lines established from J-2, J-3, or J-5 neoplasias grow in culture without specific growth factor supplements. A function for v-myc in synergism with v-raf has been established by experiments demonstrating that v-myc can abrogate growth factor requirements of IL-3- and IL-2-dependent cells and v-myc-infected fibroblasts grow in medium depleted of PDGF. A function for raf in the signal transmission pathway of growth factors has been established in studies involving raf transformation of Ki-ras-resistant cells and in microinjection analyses of ras and raf antibodies into normal, ras- and raf-transformed mouse fibroblasts. These studies suggest that raf is located downstream of ras in signal transmission. Finally, even tumors induced by the dual oncogene virus J-2 are clonal, suggesting that additional events may be required in the maintenance of the transformed phenotype. In an in vitro-derived J-2-infected myeloid cell line, we have identified a likely alteration in the c-myc proto-oncogene, a proviral insertion common to an in vivo-derived tumor.

PROJECT DESCRIPTIONNames, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

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Robert Bassin	Senior Investigator	LTIB	NCI

Objectives:

The specific contributions of oncogenes acting alone or in concert in the etiology of human neoplasias has remained an enigma, and identification of likely cellular genes which are targeted for alterations in cancer has come largely from indirect evidence. Several types of data indicate interactions between raf and myc in some human tumors, including small cell lung carcinoma, familial renal carcinoma, mixed parotid tumors, ovarian carcinoma, and adenocarcinoma of the stomach. To study synergism between raf and myc, we have directly analyzed expression of these two oncogenes in human tumor samples and investigated their synergism and function in the mouse utilizing a series of recombinant murine retroviruses carrying raf and myc family oncogenes.

Methods Employed:

RNA and DNA from human tumors was analyzed using established protocols. Standard recombinant DNA technology was utilized in the construction of new murine retroviruses, and high titer stocks of these viruses were prepared after transfection onto NIH 3T3 cells. Expression of recombinant retroviruses and cellular proto-oncogenes in induced murine tumors and transformed cell lines was assessed using standard criteria for Northern hybridization and Western blotting.

Major Findings:

We have made significant progress in evaluating the roles of raf and myc oncogenes in natural tumors and have elucidated probable functions of these two families of proto-oncogenes in normal and transformed cells. The major findings can be summarized as follows:

1. Neoplasias believed to have c-raf-1 involvement, namely those showing chromosomal alterations at or near the c-raf-1 locus on chromosome 3, band p25, have been analyzed by Northern and Western analyses for c-raf and c-myc expression. Sixty percent of small cell lung carcinoma cell lines, which characteristically have deletions of 3p, show very high levels of c-raf-1 RNA coupled with amplification of one of the myc proto-oncogene family members. In mixed salivary gland tumors, with translocation of 3p25 to 8q, one again sees very high levels of both c-raf-1 and c-myc mRNA, suggesting a complementation of these two genes in transformation of these human cells in vivo.

2. A synergism between raf and myc oncogenes in transformation in vivo and in vitro and the possible contribution of each oncogene to this complementation was examined in the mouse. The specific in vivo transforming targets of raf and myc family oncogenes, irrespective of transcriptional regulatory elements, has been established by insertion of these genes into a common Moloney leukemia virus and infection of newborn NFS/N mice. raf family oncogenes predominately induce rapid fibrosarcomas (7 week latency) in newborn mice, but also induce erythroid hyperplasia. In vitro, raf family genes cause the release of transforming growth factors from transformed fibroblastic cells. In primary hematopoietic cells, raf fails to abrogate growth factor requirements or induce growth factor secretion, but are active in immortalizing these cells in the presence of specific IL supplements. In contrast to the restricted distribution of in vivo lineages for raf transformation, infection of newborn mice with v-myc retroviruses results in a wide range of transformed lineages including hematopoietic, lymphoid, and epithelial neoplasias with a latency period dependent upon the replication efficiency of pseudotyping MuLV in newborn NFS/N tissues. Combination of v-raf and v-myc in the J-2 virus has demonstrated a synergism between these two oncogenes in terms of rapid disease induction (average latency 2 weeks) in vivo and in the immortalization of growth factor independent macrophages, myeloid stem cells, and pre B-cells in infected mouse primary bone marrow or fetal liver cultures. Lymphoid/hematopoietic cells transformed by the J-2 virus on v-myc viruses only can be readily established in culture without the addition of specific growth factors (IL) normally required by these cells. In vitro, we have demonstrated that high levels of v-myc expression can abrogate the growth factor requirements of established myeloid and T lymphoid cells for IL-3 and IL-2, respectively, and have observed that v-myc-infected fibroblasts grow readily in medium depleted of PDGF. These findings suggest that myc can act as a central relay in the signal transmission pathway of several growth factors and predicts that alterations in myc should, as observed in our studies with v-myc viruses only, transform a large number of lineages. The contribution of raf in synergism with myc has been assessed in tests for the dominance of raf in the transformation of Ki-ras-resistant revertant cell lines and in microinjection studies testing the ability of microinjected ras and raf neutralizing antibodies in blocking normal, ras-, and raf-transformed mouse fibroblasts from entering S phase. These studies have shown that raf family oncogenes, unlike several other oncogenes, are able to transform Ki-ras-resistant revertants and, when microinjected, that ras antibody will block S phase in normal, but not raf-transformed cells, whereas raf antibodies block ras-transformed and normal fibroblast DNA synthesis. These experiments suggest that either raf family oncogenes are located downstream of ras in the signal transmission pathway of growth factors for fibroblasts or that raf is a component of an alternate pathway for signal transmission of growth factors. In analogy to the Balb/3T3 system which defined two components necessary for a cell to enter S phase, "competence" signals, acting early in G1, and "progression" factors, which act late in G1, we propose that the observed synergism between raf and myc is a consequence of the constitutive firing of competence (v-myc) and progression (v-raf) signals in driving transformed cells through the G1 phase of the cell cycle.

3. We have established that even tumors induced by the dual oncogene virus J-2 are, as a rule, clonal in nature suggesting either the selective outgrowth of one infected, transformed cell over that of the other infected cohorts or that additional alterations were required for the maintenance of the transformed

phenotype. In an in vitro system involving the infection of primary hematopoietic stem cell cultures in the presence of IL-3, we have discovered a common second event, rearrangements of the c-myb proto-oncogene in two J-2 virus-transformed myeloid cell lines. In one case, the c-myb rearrangement was established to be the consequence of the insertion of a MuLV helper virus into the sixth coding exon of c-myb and resulted in expression of high levels of truncated c-myb RNA and protein. Comparison of this insertion to that of a wild-mouse ecotropic provirus insertion in an in vivo myeloid tumor established that the insertion occurred at the exact same nucleotide--the first demonstration of an exact common retroviral integration into a cellular genome. In vitro infections of this and other culture systems then represents a powerful tool where one can, at least in the case of retroviral insertion mutations, "transposon tag" likely cellular gene targets which are associated with transformation of a particular cell lineage or have a selectable function in in vitro culture.

Publications:

Blasi, E., Mathieson, B., Varesio, L., Cleveland, J. L., Borchert, P. A. and Rapp, U. R.: Selective immortalization of murine macrophages from fresh bone marrow by a raf/myc recombinant murine retrovirus. Nature 318: 667-670, 1985.

Cleveland, J. L., Jansen, H. W., Bister, K., Morse, H. C., Ihle, J. N. and Rapp, U. R.: Interaction between raf and myc oncogenes in transformation in vitro and in vivo. J. Cell. Biochem. 30: 195-218, 1986.

Ihle, J. N., Keller, J., Rein, A., Cleveland, J. and Rapp, U.: Interleukin 3 regulation of the growth of normal and transformed hematopoietic cells. In Fermisco, P., Ozanne, B. and Stiles, C. (Eds.): Cancer Cells: Growth Factors in Transformation. New York, Cold Spring Harbor Laboratory, 1985, pp. 211-219.

Ihle, J. N., Keller, J., Rein, A., Pierce, J. and Rapp, U. R.: The effects of transforming retroviruses on mast cell growth and IL-3 dependence. In Befus, D., Bienenstock, J. and Denburg, J. (Eds.): Mass Cell Heterogeneity. Hamilton, Raven Press (In Press)

Ihle, J. N., Weinstein, Y., Rapp, U. R., Cleveland, J. L. and Reddy, E. P.: Mechanisms in IL-3 regulated growth and differentiation. In Gupta, S., Paul W. E. and Fauci, A. S. (Eds.): Proceedings of the Conference on Lymphocyte Activation and Immune Regulation. New York, Plenum Press (In Press)

Morse, H. C., III, Hartley, J. W., Frederickson, T. N., Yetter, R. A., Majumdar, C., Cleveland, J. L. and Rapp, U. R.: Recombinant murine retroviruses containing avian v-myc induce a wide spectrum of neoplasms in newborn mice. Proc. Natl. Acad. Sci. USA (In Press)

Rapp, U. R., Bonner, T. I., Moelling, K., Jansen, H. W., Bister, K. and Ihle, J.: Genes and gene products involved in growth regulation of tumor cells. In Havemann, K. and Sorenson, G. (Eds.): Recent Results in Cancer Research. New York, Alan R. Liss, 1985, pp. 221-236.

Rapp, U. R., Cleveland, J. L., Morse, H. C., Frederickson, T., Holmes, K., Jansen, H. W. and Bister, K.: Synergistic oncogenes: Rapid induction of hemopoietic tumors in mice by a raf/myc recombinant virus. J. Virol. 55: 23-33, 1985.

Patents:

None

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01CPD5489-01 LVC

PERIOD COVERED

October 1, 1985 to September 30, 1986

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Rearrangement of c-raf-1 Oncogene in Human Breast Carcinoma

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Chirabrata Majumdar Expert LVC NCI

Others: Ulf R. Rapp Chief, Viral Pathology Section LVC NCI
John L. Cleveland Staff Fellow LVC NCI

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1.0

PROFESSIONAL:

0.9

OTHER:

0.1

CHECK APPROPRIATE BOX(ES)

- (a) Human subjects (b) Human tissues (c) Neither
- (a1) Minors
- (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The v-raf is the oncogene transduced by the 3611 MSV which causes fibrosarcoma in newborn mice. Using this oncogene as a probe, we have successfully characterized several raf-related cellular counterparts, in man and mice, which exhibit significant nucleotide and amino acid homology so that they can be classified under a family of oncogenes. To elucidate the role of raf oncogenes in malignancy, we investigated the possibility of rearrangements of genomic raf DNA from various tumors and transformed cell lines. The DNA from a human breast carcinoma cell showed altered restriction patterns as compared to normal DNA when probed with c-raf-1 cDNA. Based on these initial results, we constructed a lambda genomic library and screened the library with the same probe. Two overlapping positive clones were further analyzed to map the rearranged locus. These studies demonstrated that two closely spaced EcoR1 sites (approximately 200 bases apart) and an adjacent Sal-1 site, all clustered around 19.5 map unit, have been eliminated from the genome. These observations agree with the sizes of DNA fragments from the tumor cell DNA on a Southern blot analysis, where the 10.3 kb EcoR1 fragment is missing. In addition, the loss of a Sal-1 site at 19.5 map units would result in a larger Sal-1 fragment which we have confirmed by isolating a clone containing DNA from 4.4 through 22.4 map units which has no Sal-1 site. Further analysis revealed that the sequences representing exon 7 have been deleted from the genome. Thus, in these cells, the c-raf-1 oncogene is rearranged within the 5' half of the gene resulting in the deletion of an exon. Truncations within the NH2 terminal half of the raf protein generally characterize transforming versions of this gene.

PROJECT DESCRIPTIONNames, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Chirabrata Majumdar	Expert	LVC	NCI
Ulf R. Rapp	Chief, Viral Pathology Section	LVC	NCI
John L. Cleveland	Staff Fellow	LVC	NCI

Objectives:

The cellular raf oncogene (c-raf-1) consists of 17 exons and is associated with Ser/Thr protein kinase activity. The kinase domain extends from exon 10 through the coding sequence of exon 17. Our objective is the identification of human tumors or cell lines in which raf genes are activated as oncogenes. Moreover, we want to elucidate the mechanism of activation of this gene and to establish whether similar mechanisms are operative in different raf-induced malignancies. We also intend to define various functional domains of this gene to develop a model which would explain how this oncogene is regulated in normal and malignant cells.

Methods Employed:

To detect the rearrangement of the raf gene, high molecular weight DNA was extracted, digested with appropriate restriction enzymes and analyzed by Southern blot hybridization using radiolabeled c-raf-1 cDNA as the probe. The DNA sample showing an altered restriction pattern was further probed with other raf-related radiolabeled cDNAs to detect rearrangements at additional loci and to determine the appropriate probes to be used for screening the genomic library. To construct the genomic library, high molecular weight DNA was partially digested by Mbo I and then ligated with BamH I digested arms of phage lambda. The ligated reaction mixture was packaged into lambda phage by standard procedure and was screened by using c-raf-1 cDNA as the probe. About 10⁶ phages were screened and several positive clones were further purified for restriction analysis. Two overlapping clones were analyzed by restriction digestion of nick translated DNA, as well as by Southern blot hybridization with c-raf-1 cDNA probe to map the genomic region they represent, as well as to determine the site of rearrangement.

Major Findings:

The two clones used for mapping represent about 30.0 kb of over 44.0 kb of the c-raf-1 proto-oncogene. This region contains exons 2 through 13. (1) The two closely located EcoR I sites upstream of exon 7 and the adjacent Sal I site downstream have been eliminated from the genome giving rise to larger EcoR I and Sal I fragments which agree with the sizes of fragments on a Southern blot. (2) The 3.4 kb Hind III and the 1.8 kb Hpa I fragments from the tumor DNA clone, both of which normally contain the exon 7, do not hybridize with c-raf-1 cDNA. Thus, the entire exon 7 appears to be deleted in the tumor cell-derived DNA.

We have previously shown that the product of the kinase domain is minimally required for transformation by c-raf-1 DNA and speculated that the NH2 terminal truncation activates the protein as a kinase, since all the transforming variants of the raf oncogene are truncated at the amino terminus. The deletion of the exon 7 from the raf proto-oncogene may similarly activate the kinase activity of the truncated protein.

Publications:

Morse, H. C., Hartley, J. W., Frederickson, T. N., Yetter, R. A., Majumdar, C., Cleveland, J. L. and Rapp, U. R.: Recombinant murine retroviruses containing avian v-myc induce a wide spectrum of neoplasm in newborn mice. Proc. Natl. Acad. Sci. USA (In Press)

Rapp, U. R., Cleveland, J. L., Majumdar, C., Jansen, H. W., Bister, K., Morse, H. C. and Ihle, J. N.: Carcinoma induction in mice by a raf/myc recombinant murine retrovirus. In Barbacid, M. and Wigler, M. (Eds.): First Annual Meeting on Oncogenes. Frederick, Maryland, Hood College, 1985, p. 29.

Patents:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05490-01 LVC

PERIOD COVERED

October 1, 1985 to September 30, 1986

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Molecular Basics of Lentiviral Transcriptional Transactivation

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	James W. Casey	Senior Staff Fellow	LVC	NCI
Others:	David D. Derse	Guest Researcher	LVC	NCI
	Gisela Fanning-Heidecker	Visiting Fellow	LVC	NCI
	Raoul E. Benveniste	Medical Officer	LVC	NCI

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1.9

PROFESSIONAL:

1.4

OTHER:

0.5

CHECK APPROPRIATE BOX(ES)

- (a) Human subjects (b) Human tissues (c) Neither
 (a1) Minors
 (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The lentivirus equine infectious anemia virus (EIAV), displays a highly restricted cell type preference both *in vitro* and *in vivo*. Additionally, like other members of the lentivirus family, EIAV is subject to antigenic variation as evidenced by changes that occur in envelope glycoproteins during the course of infection. To further understand the restrictive host range and correlate proviral changes that occur during pathogenesis, we have molecularly cloned and sequenced an EIAV provirus. Comparison of *gag* and *pol* genes of EIAV with human immunodeficiency virus (HIV) and visna clearly establishes that EIAV is genetically related and equally divergent from these two distinct lentiviruses. We have examined the long terminal repeats (LTRs) of EIAV with respect to their ability to function as transcriptional promoters in various cellular environments. Nucleotide sequence analyses of the LTRs derived from two unique proviral clones revealed consensus transcription and processing signals. One of the proviruses possessed a duplication of a 16 base pair (bp) sequence in the CCAAT box region which was absent in the other provirus. To assess their functional activity, each LTR was coupled to the bacterial chloramphenicol acetyl transferase (CAT) gene and transfected onto various cell lines, including matched cultures of EIAV-infected and -uninfected cells. The levels of CAT activity directed by the EIAV LTRs were between 250 and 900 times greater in EIAV-infected cells compared to uninfected counterparts. Thus, EIAV expression appears to be subject to a virus-induced transactivation analogous to that recently shown to amplify expression of certain other lentiviruses including HIV. To broaden our understanding of lentivirus viral gene regulators and to acquire additional reagents to examine and detail the mechanism of lentiviral transactivation, we have begun to molecularly clone the simian lentivirus macaque immunodeficient virus (MnIV).

PROJECT DESCRIPTIONNames, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

James W. Casey	Senior Staff Fellow	LVC	NCI
David D. Derse	Guest Researcher	LVC	NCI
Gisela Fanning-Heidecker	Visiting Fellow	LVC	NCI
Raoul E. Benveniste	Medical Officer	LVC	NCI

Objectives:

Transactivation, a phenomenon in which a viral gene product acts in trans to increase its own transcription is a unique feature of lentiviruses. We have established that EIAV displays the properties of trans-activation and will focus our effort on mapping the trans-acting responsive element in the LTR and isolating the trans-acting gene. We wish to molecularly clone an additional member of the lentivirus family, macaque immunodeficient virus (MnIV), and examine the functional relationship of this virus to EIAV by detailing its mechanism of transcriptional regulation.

Methods Employed:

EIAV was molecularly cloned in lambda vectors and subcloned in plasmid vectors. DNA sequence data were obtained using the Sanger dideoxy and Maxam Gilbert chemical methods. Functional analysis of promotor strength was measured using the chloramphenicol activity transferase (CAT) assay. MnIV first stage molecular cloning was accomplished using synthetic oligonucleotides and consensus polymerase probes.

Major Findings:1. The nucleotide sequence of EIAV.

Full-length EIAV proviral DNA was cloned from an infected equine fibroblast cell line and after appropriate subcloning, the 5' half encompassing gag and pol was sequenced. EIAV, as in HIV, utilizes a tRNA^{Lys} as a primer for first strand synthesis. The initiation codon for gag gene precursors (p26, p11 and p9) begins 124 bases after the tRNA^{Lys}. The pol gene overlaps gag by 241 bases, as in HIV, so that a frameshift must occur to effect translation. The computer program ALIGN, which employs a scoring matrix and gap penalty, was used to evaluate the degree of relatedness of EIAV with visna and HIV. EIAV appears to resemble HIV, rather than visna, in the use of tRNA^{Lys} and in encoding of a p9 protein at the carboxy terminus of gag. Depending on the region of the genome used for comparison, EIAV appears to resemble HIV slightly more than visna. For example, p26 shares 30% of the AA residue with HIV, while the relationship between visna and EIAV is 24%. This pattern is observed only in subregions of gag, whereas central regions of p26 show little or no homology to any known retroviruses. The most obvious region of homology between EIAV and visna/HIV is localized to the NH₂ region of pol. Fifty-three percent of amino acid residue between 211-420 of EIAV is identical to HIV. Further, the nucleotide sequences of the region is conserved

to the extent of 60-70% and is now being used as a lentivirus-specific hybridization probe. An additional feature of EIAV which allows grouping with other lentiviruses is that its overall base composition is 38-39 mole percent G/C.

2. Subcloning, sequence analysis and assay of the promoter activity of the EIAV LTR.

Both 5' and 3' LTRs from lambda clone EIAV-12 and the 3' LTR of lambda clone EIAV-6 were sequenced and subcloned into pSV0cat for assay of promoter activity. Sequence analyses revealed that the EIAV LTRs contain the requisite transcriptional and processing signals, as well as consensus TATA boxes, CAAT boxes and polyadenylation signals AATAAAA, in the LTRs. No consensus enhancer sequences, TGG T/A T/A T/A G, or long stretches of homology with LAV/HTLV-III or visna virus were found, although two short, clustered regions of homology with visna virus were identified. Comparison of the LTRs of lambda EIAV-6 and EIAV-12 revealed a single base change, as well as a 16 bp duplication and a 4 bp insertion in the LTR of lambda EIAV-6 relative to lambda EIAV-12.

In functional assays, we observe high levels of CAT activity as directed by the EIAV LTRs in infected cells and very low levels in uninfected cells. Activities directed by the EIAV LTR in uninfected cells ranged from 2% in monkey CV-1 cells to 8% in human RD-4 cells, of the levels directed by pSV2cat (positive control). Thus, the EIAV LTR is able to function, albeit at low levels, in uninfected cells. In the matched sets of cells where one set was productively infected with EIAV, the levels of CAT activity was 250-900 times higher in the infected set than the uninfected set. The basal level of functional activity of EIAV-6 was four times higher than EIAV-12. It remains to be determined if the duplication of a 16 bp segment is responsible for this effect. EIAV LTRs were nonfunctional when cloned in the reverse orientation, substantiating the validity of the assay. In conclusion, the EIAV LTR responds to factors present in EIAV-infected cells, suggesting that an EIAV-encoded gene product is responsible for transactivation. The focus of our research will be to identify and to isolate the viral encoded trans-acting factor and the responding element in the LTR.

3. Molecular cloning of macaque immunodeficiency virus (MnIV).

The simian virus MnIV displays properties which allow a preliminary classification of the virus to the lentivirus family. The morphology, restricted host cell type range (T-cells), disease pattern, and antigenic cross-reactivity with HIV imply that it is an evolutionarily close relative of HIV. We attempt to molecularly clone MnIV in order to rigorously establish the above relationship and to understand its pattern of gene expression. A peculiarity unique to MnIV is its extremely low level of infectivity compared with type C retroviruses or HIV. Briefly, MnIV produces approximately fivefold more virus as measured by electron microscopic methods and reverse transcriptase assays, yet contain no measurable 35s or 70s RNA. The infectivity measured by end point dilution is 10^3 less than a comparable assay with HIV or type C virus. These data suggest that processing of viral RNA or packaging of the genome is uniquely regulated. To address the above question, we have constructed recombinant lambda libraries of DNA from H9 infected cells and screened these resulting clones with two unique probes. As stated in section 1, the lentiviruses contain a segment in the polymerase gene of length 271 nucleotide, which is highly conserved among all members. We have utilized the

EIAV conserved region as a hybridization probe and also synthesized an oligonucleotide of 25 bp from the P-26 region as an additional probe. Positive signals were obtained and are presently being characterized.

Publications:

Stephens, R. M., Casey, J. W. and Rice, N. R.: Equine infectious anemia virus gag and pol genes: Relatedness to visna and AIDS virus. Science 231: 589-594, 1986.

Patents:

None

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01CP05491-01 LVC

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October 1, 1985 to September 30, 1986

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

v-myc Regulation of c-myc Expression

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	John L. Cleveland	Staff Fellow	LVC	NCI
Others:	Ulf R. Rapp	Chief, Viral Pathology Section	LVC	NCI
	Mahmoud Huleihel	Visiting Fellow	LVC	NCI
	Patricia Borchert	Microbiologist	LVC	NCI

COOPERATING UNITS (if any)

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Viral Pathology Section

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TOTAL MAN-YEARS:

1.0

PROFESSIONAL:

0.5

OTHER:

0.5

CHECK APPROPRIATE BOX(ES)

- (a) Human subjects (b) Human tissues (c) Neither
- (a1) Minors
- (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Tumors induced in vivo by v-myc recombinant retroviruses and mouse cell lines from a variety of lineages infected with these viruses, which express high levels of avian v-myc, were found to be invariably associated with a lack of c-myc expression. In order to distinguish between v-myc-induced shutdown versus a differentiation-associated down regulation of c-myc expression, we have determined steady state levels of c-myc mRNA in three different cell lines in culture which express various levels of c-myc prior to infection. Extreme levels of v-myc expression (10- to 100-fold excess over c-myc) was achieved in a myeloid (FDC-P1) and a T lymphoid (CTB-6) cell line. In both lines, c-myc expression was absent in the infected cells as assessed by RNA and nuclear run-on analyses and, in the case of FDC-P1, could not be induced by growth factor (IL-3) or inhibitors of protein synthesis (to remove a labile repressor). Moreover, DNase I hypersensitive sites typical for active c-myc alleles were absent in FDC-P1 cells infected with v-myc retroviruses. In NIH 3T3 fibroblast cells, v-myc was expressed at least five times the levels of c-myc present in uninfected cells. Again, v-myc expression was associated with down-regulation of c-myc. However, in contrast to infected FDC-P1 cells, down-regulated NIH 3T3 cells could be induced for c-myc expression by treatment with anisomycin for periods corresponding to greater than three times the half-life of v-myc protein in these cells. A deletion analysis of v-myc revealed that removal of 109 amino acids from the beginning of the sequence derived from the second c-myc coding exon was sufficient to abolish its ability to down-regulate c-myc expression.

PROJECT DESCRIPTIONNames, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

John L. Cleveland	Staff Fellow	LVC	NCI
Ulf R. Rapp	Chief, Viral Pathology Section	LVC	NCI
Mahmoud Huleihel	Visiting Fellow	LVC	NCI
Patricia Borchert	Microbiologist	LVC	NCI

Objectives:

Alterations in the regulation of myc family proto-oncogene expression is believed to be involved in the etiology of a wide variety of human neoplasias. In our experimental model, we have demonstrated that c-myc transcription can be modulated by expression of high levels of v-myc from recombinant retrovirus and, moreover, that c-myc expression can be either suppressed or induced depending on the portions of v-myc present in the infecting retrovirus, a fact which suggests that v-myc indeed regulates c-myc. Our objectives are to determine the mechanism(s) controlling this regulation, identify regulatory sequence elements of myc family proto-oncogenes which respond to negative and positive transcriptional regulatory factors, and to test, using retroviral v-myc and myc family constructs, whether we can suppress altered myc expression and, hence, myc-induced transformation.

Methods Employed:

Recombinant retroviruses containing various portions of the v-myc gene (from MH2 or MC29) were constructed using standard recombinant DNA technology. High titer stock of MuLV pseudotypes of these defective retroviruses were prepared from transfected NIH 3T3 cells using established techniques. RNA analyses, nuclear run-on assays, and DNase I hypersensitivity assays of chromatin were all performed using standard protocols.

Major Findings:

The data implicating v-myc as a regulator of c-myc expression can be summarized as follows:

1. In tumors induced in vivo by v-myc recombinant retroviruses and in cell lines established from these tumors, we have observed a lack of c-myc expression irrespective of the lineage transformed including hemopoietic, lymphoid, and epithelial cells. Moreover, in vitro infections of primary hemopoietic stem cell cultures with the J-2 virus, containing both raf and v-myc oncogenes, has led to the isolation of growth factor independent B lymphoid, macrophage, and myeloid stem cell lines, all of which again do not express c-myc. These results were similar to those observed previously, mainly in Burkitt's lymphoma and mouse plasmacytomas: that high levels of expression of c-myc from an altered allele is often associated with the absence of expression from the normal allele; however, in these cases one lacks the appropriate control cell lines.

2. To be able to discriminate between a v-myc-induced shutdown of c-myc expression versus a differentiation-associated shutdown of c-myc, we first turned to infection of growth factor-dependent myeloid (FDC-P1) and T lymphoid (CTB6) cell lines, where it had been demonstrated that high levels of v-myc expression abrogated the growth factor requirements of these cells. Under this selective pressure, we were able to isolate several homogeneously infected factor-dependent lines, all of which expressed high levels of v-myc. However, these cells do not express c-myc, even though c-myc is expressed at high levels in the uninfected cells. Moreover, in the factor-independent myeloid cell line, FD/J-2, c-myc transcription was shut off at the level of transcription, as assessed by nuclear run-on assays, and was not inducible with IL-3 (unlike the uninfected FDC-P1 cell in which c-myc was inducible), even though receptors for this ligand were retained and bound iodinated IL-3. To further test the reversibility of c-myc expression, we treated factor independent cells with the protein synthesis inhibitor anisomycin to remove a labile repressor that might be involved in c-myc down-regulation. Again, c-myc expression was not inducible in these cells. Moreover, the conformation of the promoter-regulatory region of c-myc gene in factor independent cells, as assessed by DNase I hypersensitivity assay, resembled a transcriptionally silent c-myc locus. Therefore, in these factor-independent myeloid cells, c-myc transcription was indeed suppressed as a consequence of v-myc virus infection, but the gene took on the conformation of an inactive allele.

3. c-myc also became undetectable in NIH 3T3 cells upon infection with retroviruses carrying a full complement of v-myc consequences. This contrasted markedly to cells infected with the J-1 recombinant virus, containing only the first exon of v-myc, where we observed increased levels of c-myc RNA. RNA and protein analysis of the effects of deletion mutants of v-myc on c-myc expression showed that neither a derivative of J-1 containing the 54 C terminal amino acids of v-myc (J-1-Bal) nor a construct lacking only the first 109 amino acids of the second exon of v-myc were able to suppress the expression of c-myc. Moreover, the down-regulation of c-myc by v-myc in fibroblasts was reversible by treatments of anisomycin for periods up to six times the half-life of v-myc protein in these cells, suggesting an active role for v-myc not only in induction, but also in maintenance of c-myc suppression. Since in the infected fibroblast system, one can either induce or suppress c-myc expression by perturbations of the v-myc oncogene, one is therefore in a position to define positive and negative transcriptional sequences controlling expression of c-myc and to identify transcriptional or post-transcriptional regulatory factors. We have recently extended our initial observations with v-myc to include N-myc and have shown that c-myc expression is undetectable in mouse fibroblasts expressing high levels of N-myc from an expression vector. This data suggests the hypothesis that each member of the myc gene family may not only be able to autoregulate its own expression, consistent with our data on v-myc regulation of c-myc, but also that there might be a cross-regulation of the entire family by each member as expression of c-myc, N-myc, and L-myc is generally mutually exclusive in different lineages of cells.

Publications:

Cleveland, J. L., Huleihel, M., Eisenmann, R., Siebenlist, U., Ihle, J. N. and Rapp, U. R.: v-myc regulation of c-myc expression. In Kjeldgaard, J., Forchhammer, T. and Thaysen, M. (Eds.): The Alfred Benzon Symposium No. 24 on Viral Carcinogenesis. Copenhagen, Munksgaard (In Press)

Morse, III, H. C., Hartley, J. W., Frederickson, T. N., Yetter, R. A., Cleveland, J. L., Majumdar, C. and Rapp, U. R.: Tumors of newborn NFS/N mice infected with murine retroviruses containing avian v-myc. Curr. Top. Microbiol. Immunol. (In Press)

Rapp, U. R.: IL-2 and IL-3 regulated growth of lymphoid cells: Abrogation by infection with a myc/raf recombinant virus. In Melchers, F. and Potter, M. (Eds.): Proceedings of the Third Meeting on "B Cell Neoplasia". Basel, F. Hoffmann-La Roche Press, 1985, pp. 292-301.

Patents:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05492-01 LVC

PERIOD COVERED

October 1, 1985 to September 30, 1986

TITLE OF PROJECT (90 characters or less. Title must fit on one line between the borders.)

Genetic Dissection of raf and myc Oncogene Functions

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Ulf R. Rapp Chief, Viral Pathology Section LVC NCI

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COOPERATING UNITS (if any)

NIAID, NIH, Bethesda, MD (H. C. Morse)

LAB/BRANCH

Laboratory of Viral Carcinogenesis

SECTION

Viral Pathology Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701-1013

TOTAL MAN-YEARS:

1.4

PROFESSIONAL:

0.8

OTHER:

0.6

CHECK APPROPRIATE BOX(ES)

- (a) Human subjects (b) Human tissues (c) Neither
 (a1) Minors
 (a2) Interviews

SUMMARY OF WORK (Use standard un-reduced type. Do not exceed the space provided.)

The structural requirements of raf and myc oncogenes were determined for transformation, synergism, growth factor abrogation, transforming growth factor induction, and autoregulation of cellular myc genes. The minimal transforming sequences of different members of raf family genes (v-raf, c-raf, mouse and human A-raf) were determined in established fibroblast cell lines and in newborn mice by making a series of raf transducing murine retroviruses with various N and C terminal deletions. Furthermore, a series of v-myc recombinant murine retroviruses were constructed with various deletions in v-myc. We found that a deletion of 14 amino acids from the C-terminal of v-raf did not affect its transforming activity, whereas deletions of 27 and 28 or more amino acids from the C-terminus of A-raf and v-raf, respectively, abolished their transforming activity. Our results, and previous results from our lab, indicate that amino terminal truncation is involved in the oncogenic activation of raf family genes, since all the natural transforming versions of the raf genes are amino terminally truncated. To further define this region, we are constructing raf recombinant retroviruses with various deletions in the amino terminal half of c-raf and A-raf genes. All deletions that were made in the kinase domain of A-raf abolished its transforming activity indicating that this activity is absolutely required for transformation. Preliminary results of the v-myc recombinant retroviruses demonstrated a region of about 100 amino acids in the center of the gene, which is absolutely required for the negative regulation of v-myc on the expression of c-myc.

PROJECT DESCRIPTIONNames, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Ulf R. Rapp	Chief, Viral Pathology Section	LVC	NCI
Mahmoud Huleihel	Visiting Fellow	LVC	NCI
John L. Cleveland	Staff Fellow	LVC	NCI
Patricia A. Borchert	Biologist	LVC	NCI
Robert Nalewaik	Microbiologist	LVC	NCI
Michael Potter	Biologist	LG	NCI

Objectives:

The specific objectives of these studies are: (1) to test the transforming activity of A-raf (a new member of the raf gene family) in vitro and in vivo by incorporating it into murine retrovirus; (2) to define the sequences required for the transforming activity of the raf family oncogenes with special emphasis on the role of N-terminal truncation for their transforming activity. Therefore, we are incorporating these genes with their complete N-terminus into retroviruses; (3) to define the structural requirements of raf genes for induction of transforming growth factors; and (4) from previous findings in our laboratory, we know that the expression of v-myc can shut down the expression of the normal c-myc in established fibroblast and hematopoietic cells in culture. Therefore, it became important to identify the region(s) in the v-myc genome which is responsible for this shutdown, as well as to determine, by use of such mutant viruses, the structural requirements of v-myc for transformation, growth factor abrogation, and synergistic transformation in conjunction with raf oncogenes. Moreover, the v-myc mutant viruses will be used to identify determinants for nuclear localization of the protein. For this we have made myc recombinant retroviruses containing the intact and deleted forms of v-myc.

Methods Employed:

Standard recombinant DNA technology was used to construct new murine retroviruses, and ligation sites were sequenced using dideoxy and Maxam and Gilbert techniques and were computer analyzed. High titer stocks of these viruses were obtained after transfection of NIH 3T3 fibroblast cells and rescue with ecotropic Moloney leukemia or amphotrophic 4070 A helper viruses. Blot analysis of DNA and RNA, protein gel analysis and immunofluorescence with v-raf- and v-myc-specific antisera and infectious cell center and reverse transcriptase assays were used to demonstrate that these cells contain and produce the respective recombinant viruses. The tumor-inducing potential of construct viruses was determined by intraperitoneal inoculation of newborn NFS/N mice. Viruses with the same in vitro and in vivo transforming properties were recovered from tumors induced by the construct viruses. To demonstrate recombinant virus in tumors, these neoplasms were analyzed by DNA and RNA blotting, and virus-specific RNA and protein were demonstrated in cell lines established from primary tumors.

Major Findings:

The major findings are as follows: (1) We incorporated the mouse and human cellular A-raf genes (new members of the raf oncogene family, which are related to v-raf) into the gag gene of a retrovirus. These raf recombinant retroviruses transformed NIH 3T3 fibroblast cells in culture and were malignant in vivo after inoculation into newborn mice. They expressed a gag A-raf fusion protein of 75 kd in the case of mouse A-raf and 82 kd in the case of human A-raf. (2) We made various deletions into the 5' and 3' ends of different members of the raf family oncogenes, trying to identify the 5' and 3' borders of these genes necessary for transformation in vitro and in vivo. We found that a deletion of 14 amino acids from the C-terminus of v-raf did not affect its transforming activity, but deletion of 28 or more amino acids from the C-terminus of v-raf and 27 amino acids from the C-terminus of A-raf abolished their transforming activity. Removal of more than 14 amino terminal residues of v-raf also abolished its transforming activity. (3) All the transforming members of the raf family are amino terminally truncated. We made different A-raf and c-raf recombinant retroviruses with various deletions and with the complete gene in order to demonstrate any regulatory effects of the amino terminal portion of the molecule. (4) We made a recombinant retrovirus containing A-raf and v-myc in order to test synergistic effects between these two genes in comparison with the synergistic effects found in our laboratory between v-raf and v-myc. (5) We made v-myc recombinant retroviruses with various deletions in v-myc in order to: (a) identify the region(s) responsible for the negative regulation of the normal c-myc by v-myc; (b) identify the region(s) responsible for the myc protein localization into the nucleus; preliminary results showed that a region of 100 amino acids in the center of v-myc caused a more loose association of the myc protein with the nucleus; and (c) test their neoplastic activity in vivo; from previous experiments, which have been done in collaboration with Michael Potter (NCI), we know that when mice were injected i.p. with pristane and 7 days later infected with two different v-myc recombinant retroviruses (J3 [subgenomic MH2-MC29 v-myc fusion] or J5 [leuk gag-MC29 v-myc fusion]) the two viruses differed in their ability to induce specific tumors. Twenty-five percent of the mice injected with J3 developed plasmacytomas. These expressed v-myc, but not c-myc, and c-myc was not translocated (in contrast to control mice injected only with pristane, where all the plasmacytomas had c-myc translocations from chromosome 12 to 15). The rest (75%) of these J3 infected mice developed myeloid neoplasms. On the other hand, all the mice injected with J5 developed myeloid neoplasms. As a result of these experiments it became important to test the deleted v-myc recombinant in this assay, and to make additional constructs to identify the genetic determinant of the observed disease specificity.

Publications:

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Morse, H. C., III, Hartley, J. W., Frederickson, T. N., Yetter, R. A., Cleveland J. L., Majumdar, C. and Rapp, U. R.: Tumors of newborn NFS/N mice infected with murine retroviruses containing avian v-myc. Curr. Top. Microbiol. Immunol. (In Press)

Potter, M., Mushinski, J. F., Mushinski, E. B., Brust, S., Wax, J. S., Wiener, F., Babonits, M., Rapp, U. R. and Morse, H. C.: Rapid induction of plasmacytomas in mice by pristane and a murine recombinant retrovirus J-3 containing an avian v-myc oncogene. Proc. Natl. Acad. Sci. (In Press)

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Rapp, U. R., Cleveland, J. L., Brightman, K., Scott, A. and Ihle, J. N.: Abrogation of IL-3 and IL-2 dependence by recombinant murine retroviruses expressing v-myc oncogenes. Nature 317: 434-438, 1985.

Patents:

None

ANNUAL REPORT OF

BIOLOGICAL CARCINOGENESIS BRANCH BIOLOGICAL CARCINOGENESIS PROGRAM DIVISION OF CANCER ETIOLOGY NATIONAL CANCER INSTITUTE

October 1, 1985 to September 30, 1986

The Biological Carcinogenesis Branch (BCB) plans, develops, directs and manages a national extramural program of basic and applied research concerned with the role of biological agents as possible etiological factors or co-factors in cancer and on the control of these agents and their diseases; establishes program priorities, and evaluates program effectiveness; provides a broad spectrum of information, advice and consultation to individual scientists and institutional science management officials relative to NIH and NCI funding and scientific review policies and procedures, preparation of grant applications and choice of funding instruments; provides NCI management with recommendations as to funding needs, priorities and strategies for the support of relevant research areas consistent with the current state of development of individual research activities and the promise of new initiatives; plans, develops and manages research resources necessary for the conduct of the coordinated research program; develops and maintains computerized data management systems; and plans, organizes and conducts meetings and workshops to further the program objectives, and maintains contact with the relevant scientific community to identify and evaluate new research trends relating to its program responsibilities.

The objectives of the research program are accomplished through a variety of extramural support mechanisms including traditional individual research project grants (R01), program project grants (P01), new investigator awards (R23), conference grants (R13), cooperative agreements (U01), contracts (N01), small business innovative research grants (R43/44), academic enhancement awards (R15) and outstanding investigator grant awards (R35). A new NIH-wide mechanism was initiated during this fiscal year called the method to extend research in time (MERIT) award (R37). Currently, the Branch administers 424 research grants with an annual budget of approximately 73 million dollars. The research projects of the Branch divide into five main categories. Research programs on viruses with a DNA core which are known or suspected to be involved in the induction of malignant transformation are included in the DNA Virus Studies components. The Branch program component designated DNA Virus Studies I deals with research on the two main groups of large DNA viruses, the herpesviruses and adenoviruses. The program component designated DNA Virus Studies II supports research on the main groups of small DNA viruses, the polyoma, simian virus 40 (SV40), and papillomaviruses. Research dealing with RNA core viruses which are known or suspected of involvement in the malignant transformation of animal and human cells are covered by RNA Virus Studies components. The Branch program component designated RNA Virus Studies I involves research concerning murine, feline, bovine, primate, and hamster viruses. The program component designated RNA Virus Studies II incorporates research involving avian tumor viruses, pox viruses, myxoviruses, picornaviruses, hepatitis viruses, and plant viruses. The Office of the Branch Chief serves as the focal point for special studies or activities of high visibility. It is currently responsible for traditional

projects, conference grants, and cooperative agreements concerning virological studies on the Acquired Immune Deficiency Syndrome (AIDS) and Kaposi's sarcoma.

To facilitate and support these research activities the Research Resources component of the Branch is responsible for developing, allocating and maintaining the inventory of biological research resources necessary for the extramural research effort. The planning, initiating and oversight necessary to generate specific research resources are functions of the individual Program Directors who administer each of the research components within the Branch. The Research Resources component includes a data management element which is responsible for the automated retrieval and inventories of BCB resources, computer-systems planning, and automated analysis and management support. The automated inventories include the research resources virus and antisera inventory, the human and animal serum collection, and the human tissue collection.

The BCB resources payback system has been described in detail in previous reports. The payback system is one in which the recipients of resource materials or services reimburse the resource or production contractor directly for the services or materials received, based on a price schedule agreed upon in advance between the NCI and its resource contractor. The contractor in turn credits those funds received from recipients against its production costs and these are shown on monthly vouchers which it submits to the Government for payments under the contract. There are currently six resource contracts functioning in the payback mode. These include two for production of viral reagents, two for animal resources, one for specialized testing services, and one for storage and distribution of frozen biological reagents. The payback system seems to be performing as expected. The demand for high quality biological reagents not readily available from commercial sources has remained fairly constant. Reimbursement for full or partial costs of services has led to more careful use by investigators of costly resource reagents, with a subsequent reduced level of effort in several resource contracts or the termination of now unnecessary activities.

Table I focuses on mechanisms of support of extramural research and related activities in biological carcinogenesis. The total BCB grant and contract budget in FY86 is estimated to be about 74.9 million dollars. It should be noted that the Branch now administers 21 program project grants at a level of 20.5 million dollars and 26 cooperative agreements at a level of 2.42 million dollars. Of the cooperative agreements, 15 are AIDS-related research projects. Table II provides an estimate of the grant and contract support, respectively, in each of the six Branch components. The Branch currently administers 9 contracts and 424 grants. Table III summarizes the research activities initiated by the Biological Carcinogenesis Branch between 1982 and 1986. The table demonstrates the broad spectrum of research activities, funded through the traditional grant and cooperative agreement mechanisms, undertaken to stimulate activity in newly emerging areas of scientific opportunity.

During FY86, the Branch was again active in sponsoring a variety of research initiatives. These reflected emerging areas of research opportunity as well as the recompetition of a productive resource contract. Since the issuance of a Request for Applications (RFA) or a Request for Proposals (RFP) is contingent upon concept approval by the Division of Cancer Etiology (DCE) Board of Scientific Counselors (BSC) and is often preceded by a workshop, this report dis-

usses the initiatives generated during the fiscal year as individual entities rather than discussing each initiative separately under the various categories of workshop, concept and RFA. In terms of scientific endeavors, three research initiatives concerned retroviruses, two involved papillomaviruses, one dealt with human polyomaviruses, one concerned vector-mediated regulation of gene expression, and one was for a repository for viral products and virally infected material.

Three RFAs originally published by the BCB in FY85 were funded in this fiscal year. The first of these concerned basic studies on the development and assessment of retroviral vaccines. It was based on a BCB workshop held at NIH on December 10-11, 1984. The RFA was designed to stimulate both in vitro and in vivo experimental studies to develop and/or assess new retroviral vaccines. It is anticipated that four new research projects will be funded as a result of the responses received to this RFA. The second initiative concerned studies of novel exogenous and endogenous human retroviruses and was based on a discussion group which met on March 11, 1985 at NIH in Bethesda. The RFA was designed to stimulate research to isolate and characterize novel human retrovirus entities and determine their significance in human cancer. It is anticipated that four new research projects will be funded as a result of the responses received to this RFA. The third RFA was concerned with the role of human papillomaviruses (HPV) in the etiology of cervical cancer. The objective of this RFA was to stimulate basic and clinical research on the role of primary HPV infections of the cervix in the progression of cervical dysplasias to carcinomas. Seven of the 17 applications received in response to this RFA were funded during FY86.

A fourth RFA concerned with the transformation mechanisms of human polyomaviruses was issued in January 1986. This RFA was developed from the recommendations of a workshop sponsored by the Branch on oncogenic human polyomaviruses which was held on March 7, 1985. The primary objective of the RFA was to stimulate basic studies on the transformation mechanisms of human polyomaviruses and to ascertain their possible role in human malignancies. The application receipt date was July 15, 1986. Grants in response to this RFA are expected to be awarded in FY87.

As indicated in an earlier report, a program announcement concerning feline leukemia virus was issued in FY85. It was based on input from a BCB workshop held on November 29-30, 1984 at the NIH. This program announcement was designed to stimulate additional studies on the biology, immunology, and molecular biology of feline leukemia virus. Thus far the announcement has stimulated the submission of only one R01 application, but that application received a meritorious priority score and will be funded from the general grant pool in FY86.

On February 18-19, 1986, the Branch sponsored a workshop on the transformation mechanisms of papillomaviruses. The goals of this workshop were to assess the state of the art in the field of papillomaviruses and to determine whether particular areas of study within the field should be stimulated. Among the recommendations of the workshop participants currently being evaluated for presentation to the DCE Board of Scientific Counselors in FY87 are: an RFA to be focused on the relationship of papillomavirus growth/transformation to cellular differentiation and the immune response, and a small business innovative research announcement to promote development of specialized reagents (e.g., monoclonal antibodies) for HPV research.

On March 28, 1986, the DNA I and RNA I components of the Branch co-sponsored a discussion group on vector-mediated regulation of gene expression. The purpose of this discussion group was to ascertain the state of the art in the use of anti-sense RNA, in particular, for gene regulation and its possible relevance to the regulation of genes expressed in virus transformed cells. The consensus of the group was that there had not been a systematic approach to study the mechanism of action of anti-sense RNA or to investigate the construction of vectors used to express anti-sense sequences in cells. The recommendations of this discussion group will be presented at the October 1986 meeting of the DCE Board of Scientific Counselors and an RFA targeted to the specific issues thought worthy of increased support will be proposed.

As indicated in a prior report, an RFP was issued in FY85 for the competitive continuation of a contract for the maintenance of a repository for viruses, sera, reagents and tissues collected under ongoing and previous resource contracts. After peer review of the proposals received in response to this RFP, a contract was awarded in FY86 to the successful offeror.

Past research sponsored by the Branch has yielded much fundamental information on biological carcinogenesis by studying animal tumor viruses which induce cells to become malignant. These viruses provide tools for understanding how cellular genes and their products convert a normal cell into a tumor cell. Of equal importance, horizontally transmitted particulate human viruses may themselves be directly responsible for some malignant transformations in man. Investigations in the area of biological carcinogenesis have shown that viral information present as nucleic acid sequences in the genes of normal cells and replicating with them may be intimately involved in the development of cancer. This may occur through either the direct effects of viral transforming genes, through the influence of viral enhancing elements (promoters or long terminal repeats) on cellular oncogenes, or through the influence of viruses with a variety of environmental factors, such as hormones, chemicals, radiation, and the like. In the research program, attention is given to studies defining the interaction of viruses and cells in both animal and human cancers. The work stresses efforts to identify minute regions of viral nucleic acid and cell chromosomes which are responsible for malignancy, to understand the molecular pathways of viral replication, to identify virus products which may trigger the transformation of a cell to malignancy, and to understand host responses to viruses which ultimately may prevent cancer.

Recent studies have shown that a number of rapidly transforming RNA viruses have derived their ability to cause cancer in animals, and perhaps in humans, from genes found in normal cells. These genetic sequences are unrelated to those necessary for normal viral replication and have been found to produce proteins which are necessary for the initiation of the transformed state. Since the gene products result in transformation, the genes have been termed "oncogenes." Oncogenes have been found to possess the following characteristics: they are essential for certain types of transformation; they are derived from cellular genetic sequences; they have a limited number of specific targets; they act by means of their translational protein products; and they are probably limited in number (approximately 20 have been found). Currently, ongoing research seeks to continue the search for oncogenes in both animal and human tumor systems and to characterize these genes; to study human oncogenes and their relationship(s) to viral oncogenes in terms of nucleic acid homology and translational (oncogene)

products; to purify and characterize the translational gene products of these genes; to use these purified products in delineating the mechanism(s) of transformation; and to define the function(s) and mechanisms of regulation of the cellular homologs (c-oncs) of viral transforming genes. The research highlights of the past year are presented here and in greater detail in the various section reports which follow.

A number of basic studies of the mechanisms of oncogenesis by both RNA and DNA viruses are in progress. These have involved delineation of the structure and function of oncogenes and their protein products, identification of cellular targets of these products, cellular changes associated with oncogene activation, identification of viral proteins associated with transformation, and development of methods to study those oncogenic viruses that do not replicate well in standard assays.

In recent studies from Bishop's laboratory, the protein encoded by human c-myc has been isolated and characterized; it has been found to have an exceptionally brief half-life, to be located in the nucleus, and to bind to DNA. These are all properties consistent with speculation that the protein somehow regulates the expression of a battery of cellular genes. However, previous suggestions that a shut-off of c-myc is the precipitating event or is essential for the differentiation of mouse erythroleukemia cells have been refuted. Previous suggestions that myc and the adenovirus gene E1A are physiologically related has been strengthened by the construction of hybrids between the two genes; the results indicate that the two genes contain domains that complement one another to give full biological function. The biological capabilities of c-myc have been further assessed by incorporating the gene into a murine leukemia virus retroviral vector. In this form, the gene can transform established lines of rodent cells to a tumorigenic phenotype, sustaining the view that "deregulation" of c-myc can contribute to natural tumorigenesis.

Further studies from this laboratory have shown that the nucleotide sequence of the human locus of the newly described N-myc proto-oncogene has been obtained. The gene is closely related to c-myc and is expressed in a controlled manner during the course of mouse embryogenesis and in a restricted manner in adults, particularly in neural tissues. Amplification and over-expression of N-myc are common abnormalities in the advanced stages of human neuroblastoma; these measurements of N-myc appear likely to be useful in the diagnosis and management of this disease. The product of N-myc has been identified: it is a nuclear protein that has a short half-life and binds to DNA, properties consistent with membership in the myc gene family. Additionally, it has been shown that N-myc can transform cultured cells in two settings: rat embryo cells in cooperation with mutant c-Ha-ras, and established rodent fibroblasts without assistance. Both forms of transformation require vigorous expression of N-myc, but no intrinsic abnormality of the gene. These findings establish N-myc as an authentic proto-oncogene and strengthen the suspicion that its amplification can contribute to the progression of human neuroblastoma. Molecular constructs containing "anti-sense" RNA have been made which allow stable repression of gene expression in cultured cells. This work was first accomplished in pilot studies in which herpesvirus thymidine kinase was the repressed gene and has now been extended to work with N-myc in the hope of obtaining direct evidence to implicate this gene in tumorigenesis.

Using a gene transfer assay, it has been possible to demonstrate consistent activation of a c-ras gene in clinical samples of human chronic myelogenous leukemia (CML) from both chronic and blast crises phases of the disease. If confirmed by further work, this finding would represent the first time that abnormalities of a ras gene have been found consistently in one type of human malignancy.

The isolation of c-DNAs representing the complete c-erb-B gene (EGF receptor) has been reported. These will be used to make hybrid genes with the gene encoding the insulin receptor and with the gene encoding the receptor for the hematopoietic growth factor CSF1 (c-fms). These hybrids will be used in an attempt to localize the specificity of action within the various receptor proteins and to dissect elements of their biogenesis. The protein encoded by v-erb-B has been found to have intrinsic tyrosine protein kinase activity; the substrate specificity of this kinase differs from that of the src protein, another oncogene product with tyrosine protein kinase activity. The v-erb-B gene has been inserted into a murine leukemia virus retroviral vector, in which form it transforms murine fibroblasts and erythroid cells which produce presently uncharacterized hematopoietic tumors in rodents.

The causative agent of human AIDS is the HTLV-III virus (HTLV-III/LAV/ARV/HIV). Recent research has demonstrated that this retrovirus is part of a large family of lymphotropic retroviruses found throughout the world in man, primates, and other mammals. Characterization of the various members of this family of viruses and their mechanism(s) of pathogenesis should facilitate the development of preventive and therapeutic measures for this disease.

Rhesus monkeys at the New England Regional Primate Research Center suffer from a fatal immunodeficiency disease similar to human AIDS. Investigators at this Center, in collaboration with others at Harvard University, have isolated a retrovirus which is serologically related to the human AIDS virus, HTLV-III. The simian virus, which is designated simian T-cell lymphotropic virus III (STLV-III), can be experimentally transmitted to other monkeys in which it causes a fatal immunodeficiency disease. The existence of this new AIDS-like animal model may facilitate the development and testing of preventative and therapeutic approaches against the human AIDS virus in a readily available primate.

Recent studies of Essex and associates have shown that a virus antigenically related to simian T-cell lymphotropic virus (STLV-III) causes natural infections in wild caught African Green monkeys in Africa, in the apparent absence of disease. These investigators have also found that healthy humans in Senegal, West Africa are infected with a virus which is more closely related to the African Green monkey virus (STLV-III^{AGM}) than to other HTLV/LAV/HIV viruses. They designated this human virus as HTLV-IV. This unique model of silent and persistent infection by a "nonpathogenic" member of the AIDS family of retroviruses may provide information on the biological interaction of this family of retroviruses with primate hosts, including humans, and is thus important in the study of the origin of human AIDS and the development of a vaccine against AIDS.

The HTLV family of retroviruses enhance their own growth through a trans-acting factor which is encoded by the viral tat gene and which specifically interacts with the viral long terminal repeat (LTR). Gene splicing techniques were used

to excise the tat gene to determine its effect on virus replication. These studies showed that HTLV-III stopped reproducing entirely when its tat gene was deleted, and suggest that control of the AIDS retrovirus in infected individuals may be achieved through drugs that can inactivate this key virus gene.

Leukemic T-cell lines that are infected with HTLV-I or -II display large numbers of membrane receptors for interleukin-2 (IL-2) and some, if not all, of these cell lines produce IL-2. Introduction of the tat gene of HTLV-II into the Jurkat T-lymphoid cell line resulted in the induction of IL-2 receptors and IL-2 gene expression. Since IL-2 promotes T-cell growth and is involved in auto-stimulatory control of cell growth, the tat gene apparently brings about T-cell overgrowth through the combined effects of over-induction of IL-2 receptors and IL-2 gene expression.

Investigators have postulated that viral interactions may be important in the pathogenesis of AIDS. Both cytomegalovirus (CMV) and HTLV-III are immunosuppressive, although their mechanism(s) of immune modulation vary. Possible interactions between these two viruses are being investigated in an in vitro system. Initial studies have used the H9 T-lymphoblastoid cell line which supports productive infection with HTLV-III, but supports only abortive infection with most CMV isolates. Using different sequences of infection with these two viruses, it was found that the presence of HTLV-III can potentiate productive infection with CMV. In contrast, pre-infection of cells with CMV did not alter the subsequent ability of HTLV-III to productively infect H9 cells. Attempts at increasing HTLV-III production by CMV induction in a low HTLV-III producer variant (AL) of the H9 cell line have suggested some enhancement of HTLV-III under these conditions.

The discovery and isolation of the HTLV-III/LAV/ARV/HIV lymphotropic family of retroviruses, and significant basic research accomplishments such as those noted above, have effectively served to clarify the etiological aspects of the AIDS syndrome. Therefore, major emphasis is now being directed to prevention and/or control measures. The NCI is approaching AIDS vaccine development from two standpoints. The first is an immediate, concerted and targeted effort mediated through the Office of the Deputy Director, NCI, which is primarily utilizing personnel and facilities located at the NCI Frederick Cancer Research Facility. This effort concentrates directly on the preparation of a vaccine to the HTLV-III virus, the presumed etiologic agent of AIDS, using currently available vaccine technologies. These activities include the production of virus materials for purification of antigen; purification of specific antigenic complexes; and assays for response to these reagents, including primate responses. A second, concurrent activity which the NCI has initiated is a long-term basic research effort to generally explore the use of new technologies in vaccine development to retroviruses. This second effort is being administered through the BCB and involved the issuance of RFA 85-CA-20, "Basic Studies on the Development and Assessment of Retroviral Vaccines," referenced above. This RFA was designed to stimulate investigator-initiated traditional research projects emphasizing basic studies on the development of appropriate vaccines against known or suspected pathogenic/oncogenic retroviruses of man. The knowledge gained from the studies to be funded under this RFA should facilitate the eventual production of a safe and effective vaccine for the HTLV-III virus and will also be important if any problems develop in the directed approach to vaccine development which is currently underway.

Human papillomaviruses (HPVs) have recently been demonstrated to be strongly associated with cervical cancer and other anogenital malignancies. Investigators have identified HPV DNA in many pre-malignant, malignant and metastatic lesions in man. HPVs, therefore, are becoming an important area of tumor virus research. However, two major barriers to research on HPVs have been the lack of good model systems to study transformation and the lack of a permissive cell system to grow HPVs in the laboratory. A novel strategy has produced a mouse/human model which will allow virus replication, readily produce benign tumors, and may provide the basis of a true malignant transformation assay. The model uses normal human epidermal tissues (cervical, laryngeal, skin, or neonatal foreskin) which are exposed in vitro to a human condyloma (genital wart) extract containing HPV type 11. Small segments of these tissues are then placed under the renal capsule of nude mice. In the mice, the tissue exposed to condyloma extract develops into masses with all the histological and biochemical characteristics of a human condyloma, a benign tumor. All epidermal tissues tested in the mouse/human model showed evidence of this benign transformation, although there was a clear tropism for anogenital tissues. Interestingly, human neonatal foreskin produced the most extensive growths which practically effaced the mouse kidney. HPV-11 has been extracted from these transformed foreskin grafts. Sufficient virus was obtained to infect a new generation of normal neonatal foreskins. Thus, it will now be possible to produce HPV-11 and possibly other HPVs in the laboratory, ensuring an adequate supply for experimental purposes. These results also demonstrate for the first time that HPV can transform human tissue to a condylomatous state under laboratory conditions. Studies are now underway to extend this model to attempt to produce malignant transformation by using both serial passages of infected tissues in nude mice and treatment of the tissues with various chemical and viral co-carcinogens.

HPV-16 is another HPV implicated in the etiology of human cervical carcinoma. Investigators are beginning to elucidate the viral functions involved in transformation by the HPV family of viruses. Studies on HPV-16 DNA containing cells have identified RNA transcripts for the E6 and E7 open reading frames in both established transformed human cell lines and in tissues from cervical carcinomas. The E7 transcripts were the most abundant transcripts in these transformed cells. These results suggest that the E7 and possibly the E6 gene products of HPV-16 may be transforming proteins. Their persistence in established transformed cells over many generations tends to support a role for E7 and/or E6 proteins in the maintenance of the transformed state. It is expected that more HPV proteins will be identified and characterized in the near future.

Recent studies are defining the antigens that may be important for the development of an Epstein-Barr virus (EBV) vaccine. This virus is associated with Burkitt's lymphoma and nasopharyngeal carcinoma as well as with monoclonal and polyclonal lymphoproliferative disease in immunocompromised patients. A plasma membrane protein, designated LMP (latent membrane protein), and found in latently infected cells has been demonstrated to elicit a T-cell response in infected individuals. LMP may also play a role in lymphocyte transformation. When LMP is expressed in NIH 3T3 cells or in Rat-1 cells, it induces some of the changes associated with cell transformation, such as changes in cell morphology and ability to grow in media containing low amounts of serum. The changes in NIH 3T3 cells are less dramatic than those in Rat-1 cells. Rat-1 cells expressing LMP frequently form mounds in monolayer culture, have a high cloning efficiency in soft agar, and are tumorigenic in nude mice. Other antigens important

in the development of a vaccine are the principal components of the outer surface of EBV virions. These are glycoproteins of 350, 220 and 85 kilodaltons; gp350 and gp220 appear to mediate virus adsorption to lymphocytes, since liposomes containing these proteins prevent virus adsorption. The genes encoding gp350 and gp220 map to the same DNA fragment. A rabbit antiserum prepared to the nonglycosylated polypeptides expressed in *E. coli* was capable of immunoprecipitating both glycoproteins and also reacted with the plasma membranes of cells that were replicating virus; this antiserum also neutralized the virus, particularly after the addition of complement. This is the first demonstration that the primary amino acid sequence of gp350 and gp220 has epitopes which can induce neutralizing antibody. Thus, this polypeptide or other similar nonglycosylated polypeptide could be used to stimulate an immune response to EBV gp350 and gp220. The fact that nonglycosylated, bacterially expressed polypeptides can elicit neutralizing antibodies is important because these antigens could be used as a nucleic acid-free vaccine and thus avoid concerns about the potential latency caused by a live EBV vaccine.

Nucleic acid hybridization technology is being explored as a means of detecting the presence of pathogens not readily detected by classical virological techniques. EBV is an example of an agent that has proven difficult to recover from clinical samples, such as saliva. A new assay for the presence of EBV DNA was developed by using a cloned EBV DNA probe (BamHI-W fragment). The assay has good specificity and reasonable sensitivity. In contrast to the lymphocyte transformation assay, which is qualitative and takes three to eight weeks to complete, the hybridization assay was semiquantitative and yielded results in 72 hours. The availability of this assay should facilitate assessment of the effects of radiation, chemotherapy, and antivirals on EBV in vivo and perhaps shed light on the possible role of the virus in the occurrence of monoclonal and polyclonal lymphoproliferative disease in immunocompromised patients.

Thus, the BCB has supported a variety of studies on both RNA and DNA viruses. These studies have demonstrated novel mechanisms by which some of these agents cause oncogenic transformation and/or cancer. In addition, a number of new scientific initiatives have been developed, and there is an increased appreciation of the role of papillomaviruses as etiologic agents in some human malignancy. Although the seminal questions of how viral oncogenes transform cells and how cellular oncogenes may be related to human cancer have yet to be answered, the research activities carried out by the BCB are providing the fundamental information necessary to their ultimate resolution.

TABLE I

BIOLOGICAL CARCINOGENESIS BRANCH

Extramural Activities - FY 1986 (Estimated)

	No. of Contracts/Grants	\$ (Millions)
Research Contracts	1	0.0
Research Grants	424	72.77
Traditional Project Grants (345 grants; \$44.17 million)		
Conference Grants (9 grants; \$0.03 million)		
Academic Enhancement Awards (1 grant; \$0.07 million)		
New Investigator Research Grants (11 grants; \$0.51 million)		
Outstanding Investigator Grants (9 grants; \$4.48 million)		
Method to Extend Research in Time Awards (MERIT) (2 grants; \$0.58 million)		
Program Project Grants (21 grants; \$20.51 million)		
Cooperative Agreements (26 grants; \$2.42 million)		
Research Resources Contracts	8	2.12
TOTAL	433	74.89

TABLE II

BIOLOGICAL CARCINOGENESIS BRANCH

Contracts and Grants Active During FY 1986

	FY 86 (Estimated)			
	CONTRACTS		GRANTS	
	<u>No. of Contracts</u>	<u>\$ (Millions)</u>	<u>No. of Grants</u>	<u>\$ (Millions)</u>
DNA Virus Studies I	1	0	96	18.39
DNA Virus Studies II	0	0	94	16.52
RNA Virus Studies I	0	0	113	18.32
RNA Virus Studies II	0	0	99	17.10
Office of the Branch Chief	0	0	22	2.44
Research Resources	8	2.12	0	0
TOTAL	9	2.12	424	72.77

TABLE III
 BIOLOGICAL CARCINOGENESIS BRANCH
 Research Initiatives 1982-86

<u>Title</u>	<u>Date Workshop</u>	<u>Date BSC Review</u>	-----AWARDS 1ST YEAR----		
			<u>FY</u>	<u>No.</u>	<u>Total Dollars</u>
NIH-NCI-DCT-CTRP-82-13 (COOP) Studies of Acquired Immune- Deficiency Syndrome (KS & Opportunistic Infections)	-	May 82	83	5	\$ 962,575
NIH-NCI-DCCP-82-18 (RFA) Hepatitis B Virus and Primary Hepatocellular Carcinoma	May 82	Sept 82	84	8	\$1,073,037
NIH-NCI-DCCP-BCB-83-3 (COOP) Infectious Etiology of AIDS and Kaposi's Sarcoma	-	Feb 83	84	11	\$1,537,613
NIH-NCI-DCE-BCB-84-19 (COOP) Studies on Bovine Leukemia	May 83	Mar 84	85	4	\$ 380,758
NIH-NCI-DCE-BCB-84-27 (COOP) Studies on Human T-cell Leukemia & Lymphoma Virus Types I & II	Apr 84	May 84	85	7	\$ 690,272
NIH-NCI-DCE-85-10 (RFA) The Role of Human Papillo- mavirus in the Etiology of Cervical Cancer	Jun 84	May 84	86	7	\$ 763,074
NIH-NCI-DCE-85-20 (RFA) Basic Studies on the Development and Assessment of Retroviral Vaccines	Dec 84	Feb 85	86	4	\$ 650,691*
NIH-NCI-DCE-85-21 (RFA) Studies on Novel Human Exogenous and Endogenous Retroviruses	Mar 85	May 85	86	4	\$ 528,774*
NIH-NCI-DCE-86-07 (RFA) The Transformation Mechanisms of Human Polyomaviruses	Mar 85	Oct 85	87		
Transformation Mechanisms of Papillomaviruses (RFA)	Feb 86	Oct 86	88		
Anti-sense RNA (RFA)	Mar 86	Oct 86	88		

* Estimated funding

OFFICE OF THE BRANCH CHIEF

GRANTS ACTIVE DURING FY86

<u>Investigator/Institution/Grant Number</u>	<u>Title</u>
1. BASILICO, Claudio New York University 5 U01 CA 37295-03	Molecular Biology of AIDS Related Tumors
2. DE NORONHA, Fernando Cornell University 5 R01 CA 37742-02	An AIDS Model
3. DESROSIERS, Ronald C. Harvard University 5 R01 CA 38205-03	Type D Retroviruses and Macaque Immunodeficiency
4. ESSEX, Myron E. Harvard University 5 U01 CA 37466-02	Association Between HTLV and AIDS
5. FINBERG, Robert W. Dana-Farber Cancer Institute 5 U01 CA 34979-03	Animal Models of AIDS
6. FOX, Fred University of California (Los Angeles) 1 R13 CA 41709-01	Conference on Viruses and Human Cancer
7. GARDNER, Murray B. University of California (Davis) 5 U01 CA 37467-03	Simian Acquired Immunodeficiency Syndrome: A Model for Human AIDS
8. GERMAN, James L., III New York Blood Center 5 U01 CA 37327-03	Chromosome Mutation in the Pathogenesis of AIDS
9. HASELTINE, William A. Dana-Farber Cancer Institute 1 R01 CA 42098-01	Molecular Viology of the AIDS Virus HTLV-III
10. HAYWARD, Gary S. Johns Hopkins University 5 U01 CA 37314-03	Interaction of EBV and CMV in AIDS and Kaposi's Sarcoma
11. HIRSCH, Martin S. Massachusetts General Hospital 2 R01 CA 35020-04	Viruses, Acquired Immuno- deficiency, and Kaposi's Sarcoma

12. HOOVER, Edward A.
Colorado State University
1 R01 CA 43216-01
Pathogenesis of Feline Leukemia
Virus Induced AIDS
13. MC DOUGALL, James K.
Fred Hutchinson Cancer
Research Center
5 U01 CA 37265-03
Cytomegalovirus in AIDS and
Kaposi's Sarcoma
14. MULLINS, James I.
Harvard University
5 U01 CA 34975-03
Retroviruses and AIDS
15. POIESZ, Bernard J.
State University of New York
Upstate Medical Center
5 U01 CA 37478-03
Acquired Immunodeficiency
Syndrome: Association
with HTLV
16. ROSENTHAL, Leonard J.
Georgetown University
5 U01 CA 37259-03
Role of HCMV in KS Associated
with AIDS
17. SCHOOLEY, Robert T.
Massachusetts General Hospital
5 U01 CA 37461-03
Human T-Cell Leukemia Virus;
Virus-Host Interactions
18. SUMAYA, Ciro V.
University of Texas Health
Sciences Center (San Antonio)
5 U01 CA 37477-03
Epstein-Barr Virus and
Chromosomal Aberrations in
in AIDS
19. VOLBERDING, Paul A.
University of California
(San Francisco)
5 U01 CA 34980-03
Studies of Acquired Immune
Deficiency Syndrome
20. VOLSKY, David J.
University of Nebraska (Omaha)
5 U01 CA 37465-03
Studies of the Viral Etiology
of AIDS
21. YOHN, David S.
International Association for
Comparative Research on Leukemia
and Related Diseases (Columbus, OH)
1 R13 CA 39923-01
XII International Symposium on
Comparative Leukemia Research
22. ZAIA, John A.
City of Hope National Medical Center
(Duarte, CA)
5 U01 CA 34991-03
Role of CMV in the Acquired
Immunodeficiency Syndrome

SUMMARY REPORT

DNA VIRUS STUDIES I

The DNA Virus Studies I component of the Branch involves research on two groups of large DNA viruses, the herpes- and adenoviruses. In this component extramural research is supported by both grants and contracts. There are 96 research grants with an estimated total funding level of 18.39 million dollars. These include the traditional research grants, program project grants, conference grants, new investigator grants, and a MERIT award. The major research emphasis lies in studies of the mechanism(s) of viral transformation, which include genome structure, function and expression (78%); and virus-cell interaction (22%). In terms of the viruses being studied, 30% involve herpes simplex virus (HSV), 27% involve Epstein-Barr virus (EBV), 9% involve cytomegalovirus (CMV), 7% involve other herpesviruses, and 27% involve adenoviruses. An applied research contract which concerns the development of indicator methods for the diagnosis and prognosis of nasopharyngeal carcinomas was completed in FY86.

Investigators supported by this program are attempting to elucidate the mechanism(s) of transformation of herpes- and adenoviruses by a variety of approaches, including localization of transformation function(s) to specific sequences of the viral genome, elucidation of the function(s) of individual viral genes, and determination of the mechanism(s) of regulation of synthesis of viral gene products.

Most members of the herpesvirus family can transform cells *in vitro* and all of them can establish latent infections in man and animals. Moreover, many of the herpesviruses have been suspected of having a role in tumor induction in man, either directly or as cofactors. Studies designed to identify the regions of the herpesvirus genome involved in carcinogenesis have used several tactics. These include investigation of morphological transformation by these viruses in cultured cells and induction of transformation by transfection of cell cultures with fragments of viral DNA.

The transforming region of Epstein-Barr virus is under investigation. Epstein-Barr virus is remarkably efficient in its ability to infect human B lymphocytes and immediately induce sustained proliferation. The virus-infected lymphocytes can be cloned and grown as continuous cell lines. The cells are tumorigenic in the brains of nude mice. Most infected lymphocytes maintain the complete virus genome as a multicopy episome; in a few cell lines most or all of the virus genome is integrated into site(s) in the cell's chromosome complement. Despite the continued presence of the complete virus genome, the infection is largely latent with certain virus genes being uniformly expressed. Because of the efficiency and immediacy with which EBV transforms lymphocytes, it is presumed that expression of these virus genes is necessary for the initiation and maintenance of lymphocyte transformation. Several of the virus genes have recently been defined. The role of each of these genes in maintaining latent virus infection and lymphocyte proliferation can now be investigated. Using transfection assays in which the viral DNA encoding a particular gene is introduced into cells in an expression vector, it is possible to test individual polypeptides for their ability to alter the *in vitro* behavior of cell lines or to alter the tumorigenic potential of cell lines when tested in nude mice.

Four different EBV encoded messenger RNAs (mRNAs) have been reproducibly detected in all latently infected cells. These RNAs are largely encoded by DNA domains which are widely separated in the EBV genome. They are designated as latency and growth transformation genes LT1, LT2, LT3 and LT4 based on the chronological order with which they have been defined. It has been recently determined that the LT1, LT2, and LT4 genes each encode one of the components of the EBV-induced nuclear antigen (EBNA) complex, EBNA2, EBNA1, and EBNA3, respectively. The EBNA protein complex was originally identified by immunofluorescence studies of latently infected cells using EBV immune human sera and was originally thought to represent the product of a single virus gene. It now appears that there are at least four EBNA proteins, each encoded by a different portion of the viral genome. At least one of the EBNA proteins is present in all EBV infected lymphocytes, including Burkitt's lymphoma (BL) lymphoblasts; and in all nasopharyngeal carcinoma (NPC) cells. Recently, a fourth component of the EBNA complex, EBNA4, has been identified. The LT3 gene encodes a latent infection membrane protein (LMP). A fifth region of the EBV genome is transcribed in latent infection to yield two small nonpolyadenylated RNAs called EBERS (EBV-encoded small RNAs) which are similar to the adenovirus VA (virus associated) RNAs in size, in their transcription by RNA polymerase III and in their ability to replace VAI and VAII in adenovirus replication systems. It is speculated that these small RNAs may also function in maintaining the latent state (40,41,50,72).

The LT1 region was provisionally identified as encoding the EBNA2 protein by indirect demonstration in that a unique EBV isolate, P3HR-1, contained a deletion in the U2 region, did not produce EBNA2 and could not transform cells. Subsequently, examination of the polypeptide synthesized from the LT1 mRNA transcripts demonstrated that EBNA2 protein was encoded in the LT1 region. The gene for EBNA2 has been incorporated into expression vectors and used to transfect Rat-1 cells. Expression of EBNA2 in this cell line did not affect contact inhibition, anchorage dependence or tumorigenicity in nude mice. Nevertheless, a number of observations indicate that LT1 is a key gene in EBV induction of cell growth transformation. First, P3HR-1 virus, which is deleted in the U2 domain, is incapable of initiating transformation. Transformation positive recombinants made between P3HR-1 and other EBV isolates all have regained a U2 domain. Second, preliminary studies involving transfection of lymphocytes with a DNA fragment which included the U2 open reading frame resulted in transient induction of DNA synthesis. Finally, the U2 domain varies among EBV isolates: many EBV isolates from Africa have a variant U2 domain and transform cells poorly in vitro. Such transformed cell lines also grow poorly in culture (40).

The LT2 gene appears to encode the EBNA1 protein. An important function of the LT2 gene has been defined by showing that a fragment of EBV DNA, designated "ori P," is necessary to maintain episomal DNA in latently infected cells and that EBNA1 is the only EBV protein necessary to permit the survival of episomes containing the ori P EBV DNA segment. The carboxyl part of EBNA1 specifically binds to defined sequences in the ori P region. Analysis of EBNA1 in latently infected cells indicates the presence of several isoelectric forms. The more acidic forms bind DNA less well, suggesting that phosphorylation may regulate functional EBNA1 activity (30,50,83, DNAI1 component reference 90, RNAII component reference 87, Office of the Branch Chief (OBC) component reference 10).

The LT4 gene appears to encode the EBNA3 antigen which is not antigenically related to either EBNA1 or 2. The RNA for this 140 kilodalton polypeptide was recently shown to be encoded in part by an open reading frame in the BamHI-E DNA fragment (40,41).

The LT3 gene encodes a plasma membrane protein, designated LMP (latent membrane protein), found in latently infected cells. The role that this protein plays in the immune response to EBV and in inducing transformation is just beginning to be delineated. T-cell immunity to the protein has been directly demonstrated by release of migration-inhibition factor on exposure of T-cells from latently infected individuals to the carboxyl 220 amino acids of the membrane protein. Furthermore, antibody to the LMP protein inhibited the reaction of T-cells with membranes of EBV infected cells.

LMP does not resemble the membrane proteins of acute transforming retroviruses which are partially homologous to growth factors, growth factor receptors, or G proteins. Rather, it resembles a protein with multiple membrane spanning domains such as the rhodopsins, acetylcholine receptor, calcium pump protein and erythrocyte membrane band 3. Since most of these proteins are believed to function in ion transport, the LMP may have a similar function, as transformed cells are known to have altered ion permeability. Calcium, in particular, affects lymphocyte proliferation. The markedly acidic carboxyl terminus of this protein is also likely to have important biological properties by virtue of its potential ability to bind to cellular cytoskeletal or enzymatic components, or it may possess ATPase or phosphoprotein kinase activity. An alternative possibility suggested by some recent observations on rhodopsin is that LMP could affect the activity of another membrane protein such as GTP binding protein, which could in turn have a *ras* oncogene-like effect. LMP has been expressed in NIH 3T3 cells and Rat-1 cells. In both types of cell, LMP expression results in a slight change in cell morphology and better cell growth in media containing low serum. NIH 3T3 cells into which the LMP gene has been introduced by an expression vector do not grow in soft agar and are not tumorigenic in nude mice. In contrast, Rat-1 cells expressing LMP frequently form piles in monolayer culture, have a high cloning efficiency in soft agar, and are tumorigenic in nude mice (40).

The biochemical mechanisms that maintain EBV latency and that are modulated upon reactivation are not understood. Findings in a series of recent experiments indicate that a form of rearranged and deleted EBV DNA, designated as heterogeneous or het DNA may provide clues about the nature of viral genes and gene products that play a role in latency of EBV. The het DNA was found in a cell line designated P3J-HR-1 which spontaneously synthesized considerable amounts of EBV. It was initially determined that het DNA was not associated with all cells of this line and that cellular subclones lacking het DNA could be readily isolated. Those cell clones which lacked het DNA spontaneously synthesized small amounts of virus, although much more virus could be recovered after induction with phorbol ester. Virus released from P3J-HR-1 cells did not immortalize lymphocytes, a defect which correlated with a sizeable genomic deletion. Instead, P3J-HR-1 virus is measured by its capacity to induce early antigens (EA) in Raji cells (which already contain an EBV genome). This assay thus detects the transition from latent infection to productive infection. Subcloned P3J-HR-1 virus without het DNA did not cause EA expression in Raji cells. A rare P3J-HR-1 subclone with large amounts of het DNA spontaneously

released virus that caused EA expression in Raji cells, Thus, spontaneous virus production, the ability to induce EA in Raji cells, and the presence of het DNA are linked. Recent studies have demonstrated that one of the three fragments of this rearranged DNA, when cloned into recombinant plasmids and used to transfect cells, can activate expression of several polypeptides from a latent viral genome. The 33 kilodalton protein that is the product of the active fragment (designated WZhet) is thus likely to be responsible for the termination of latency. While the het DNA has the structural properties of defective viral DNA, its biotype is activating rather than interfering, the usual biotype for defective DNA (50, DNAlI component reference 90).

In addition to the recent definition of LMP as a potentially important immunogenic EBV antigen, the use of expression vector systems has made possible the delineation of viral sequences encoding several other EBV antigens which are involved in the immune response to viral infection. These antigens are diagnostic for both the infectious and neoplastic diseases associated with EBV. Since the initial assays for many EBV antigens were based on immunofluorescence tests, they were not only tedious to carry out, but measured complexes of antigens occurring at different times during the infectious or latent cycle, rather than individual polypeptides. The early antigen (EA) complex of EBV can be demonstrated in both producer cell lines or in latently infected cell lines (after the latter cells are superinfected or induced with phorbol esters or sodium butyrate). The early antigen class is divided into the predominantly nuclear, diffuse antigens (D), and the cytoplasmic, restricted (R), subsets which are distinguished by their cellular localization and their differential susceptibility to methanol fixation. Investigators now have used a bacterially synthesized product of one of the open reading frames in the BamHI-M (BMRF1) fragment to generate a specific antiserum which has enabled them to characterize the product of this open reading frame as a 48/50 kilodalton EA(D) polypeptide (23,31).

The principal components of the outer surface of EBV are glycoproteins of 350, 220 and 85 kilodaltons. Polyclonal and monoclonal antibodies against these proteins react with the surface of productively infected cells and neutralize virus suggesting that these antigens are important in the humoral immune response to EBV infection. The gp350 and gp220 probably mediate virus adsorption to lymphocytes, since liposomes containing these proteins prevent virus adsorption. It was known that the gp350 and gp220 mapped to the same DNA fragment and that the mRNAs encoding them had the same 5' and 3' ends. A rabbit antiserum was prepared to the EBV-beta-galactosidase fusion protein expressed in *E. coli*. This rabbit antiserum was capable of immunoprecipitating both glycoproteins and also reacted with the plasma membranes of cells that were replicating virus; the antiserum also neutralized the virus, particularly after the addition of complement. This is the first demonstration that the primary amino acid sequence of gp350 and gp220 has epitopes which can induce neutralizing antibody. Thus, this protein or other similar nonglycosylated proteins could be used as a vaccine component to stimulate an immune response to EBV gp350 and gp220. The fact that nonglycosylated, bacterially expressed polypeptides can elicit neutralizing antibodies is important because these antigens could be used as a nucleic acid-free vaccine and thus avoid concerns about the potential latency caused by a live EBV vaccine (40,72,88).

The role of the lymphotropic herpesviruses, EBV and CMV, in AIDS is still being defined. Investigators have postulated that viral interactions may be important in the pathogenesis of AIDS. Both CMV and the human T-cell lymphotropic virus type-III (HTLV-III) are immunosuppressive, although their mechanism(s) of immune modulation vary. Interactions may consist of complementary immunosuppressive effects or direct intracellular mechanisms, e.g., complementation, phenotypic mixing or recombination. These possibilities are being investigated using several approaches. Initial studies have used the H9 T-lymphoblastoid cell line which supports productive infection with HTLV-III, but only supports abortive infection with most CMV isolates. Various regimens of infection were employed. It was found that if cells were simultaneously exposed to CMV and HTLV-III or were preinfected with HTLV-III before exposure to CMV, infectious CMV was produced as detected by cytopathic effects and antigen detection after passage onto human fibroblast cell lines. This result demonstrates that the presence of HTLV-III can potentiate productive infection with CMV. In contrast, pre-infection of cells with CMV did not alter the subsequent ability of HTLV-III to productively infect H9 cells. Attempts at increasing HTLV-III production by CMV induction in a low producer variant (AL) of the H9 cell line have suggested some enhancement of HTLV-III under these conditions (34).

In contrast to the situation with herpesviruses, the E1 genome region of adenoviruses has been determined to contain the genetic information necessary for transformation by adenoviruses. Two subregions, E1A and E1B, apparently each have important roles in this process. The need for two genome fragments supports the overall mechanism of transformation of cells in vitro by oncogenes which has been considered as a two step process in which primary cells are first immortalized (by genes such as myc or polyoma large T) to produce established cell lines of indefinite proliferative capacity, and then further transformed (by genes such as ras or polyoma middle T) to produce cells of altered morphology that are less susceptible to arrest of growth by contact inhibition or other factors. Application of this model to the transformation of rodent cells in culture by human adenoviruses identified the E1A gene products as necessary and sufficient for the immortalization step. E1B gene products have been thought to be necessary for full transformation, although more recent experiments in which E1B genes have been expressed in established cell lines in the absence of E1A imply that E1B is not sufficient for the second step. Moreover, the morphology and other properties of cells transformed by the entire E1 region depends in part on which of two different adenovirus serotypes is used as a source of the E1A genes. Recent studies have used a series of plasmids containing E1A and/or E1B genes to transfect NIH 3T3 cells and to study the effects of expression of various amounts of particular E1 proteins on the transformation process. These experiments indicate that the level of expression of E1A gene products has profound effects on cellular properties; when expressed at high levels, E1A proteins alone are able to induce many of the attributes of morphological transformation that have been previously assigned to E1B proteins (45).

Although transformation following infection by adenoviruses is inefficient, the high percentage of morphologically transformed colonies seen with appropriate E1 constructs in cotransfection experiments demonstrates that adenovirus E1 genes are capable of efficient transformation when taken up by cells capable of expressing foreign genes and are thus dominant transforming oncogenes. Consequently, the limiting factor in transformation by adenovirus is likely to be viral functions involved in inserting viral DNA into the cell in a way in which

it can be efficiently expressed, rather than the functioning of the E1 oncogenes once expressed. Comparison of the capacity to transform cells of plasmids in which adenovirus E1A genes are controlled by homologous adenovirus (Ad) upstream sequences and by plasmids controlled by heterologous metallothioneine (MT) upstream sequences demonstrated that the limited transformation capability previously ascribed to E1A originates from the regulation of E1A expression, not from intrinsic limitations of E1A gene products. Thus, an adenovirus E1A construct with a homologous Ad upstream sequence exhibited very limited capacity for morphological transformation, while an MT E1A construct was as efficient in transformation as an Ad E1AE1B or MT E1AE1B construct. Additional studies in which the levels of E1A mRNA were studied also confirmed the fact that the presence of E1B or MT sequences seems to enhance the expression of E1A gene products. A major implication of these results is that the E1B gene products regulate the level of E1A expression, the reverse of the well-established role of E1A in enhancing the expression of E1B (45).

Studies on the regulation of adenovirus genes have not only enhanced knowledge on transformed cells, but also on the overall process of gene regulation in cell growth and differentiation. In complex transcriptional units, a nuclear RNA precursor can yield alternative mRNAs and proteins depending on the selection of the RNA 3' end site and/or splice site. Most transcription units in DNA and RNA tumor viruses are complex, and more than a dozen cellular complex transcription units have now been identified. Complex transcriptional units are important because they compress genetic information and because they provide for gene regulation at the level of RNA processing. In some cases, the choice of the RNA 3' end or splice site is a function of the differentiated state of the cell. In other cases, the 3' end and splice site selection remains constant; nevertheless, this selection controls the amount of each mRNA and protein product. Investigators have developed a novel class of deletion mutants in the adenovirus E3 region. Investigators have been able to identify a region that resulted in the enhancement of splicing of one of the nine mRNAs that are encoded by the adenovirus E3 region. This was the first demonstration of the enhancement of the use of a particular splice site. It is hypothesized that these mutants have defined a *cis*-acting region that affects the efficiency of the 951 5' splice site. These results may be relevant to the control of differential splicing in complex transcription units of both viruses and cells (94).

Nucleic acid hybridization technology is being explored as a means of detecting the presence of pathogens not readily detected by classical techniques. EBV is an example of an agent that has proven difficult to recover from clinical samples, such as saliva, despite the fact that this virus is ubiquitous in human populations. The virus is important both in infectious disease and neoplastic disease, being associated with mononucleosis, Burkitt's lymphoma and nasopharyngeal carcinoma, as well as with polyclonal and monoclonal lymphoproliferative syndromes. A new assay for the presence of EBV DNA was developed by using a cloned EBV DNA probe (BamHI-W fragment). When compared to the current and more tedious lymphocyte transformation assay on the same saliva samples, the sensitivity was 75% and the specificity was 97%. In contrast to the lymphocyte transformation assay which is qualitative and takes three to eight weeks to complete, the hybridization assay was semiquantitative and yielded results in 72 hours. The availability of this assay should facilitate assessment of the effects of radiation, chemotherapy, and antivirals on EBV *in vivo* and perhaps

shed light on the possible role of the virus in the occurrence of monoclonal and polyclonal lymphoproliferative disease in immunocompromised patients (OBC component reference 10).

Because of their large size, structure and host range, the DNA genomes of HSV 1 and 2 have the potential to be used as vectors of genetic material. Recently, new techniques have been developed to make specific insertions or deletions of genetic information at specific sites of the HSV genome. At the same time, the regions of the viral genome involved in the undesirable property of neurovirulence have been elucidated. Thus, the prospect of using herpesviruses as vectors for gene therapy is becoming more promising (71,72,90).

On March 28, 1986, the DNA I and RNA I components of the Biological Carcinogenesis Branch co-sponsored a discussion group on "Vector-Mediated Regulation of Gene Expression." The purpose of this discussion group was to ascertain the state of the art in the use of anti-sense RNA, in particular, for gene regulation and its possible relevance to the regulation of genes expressed in virus transformed cells. The consensus of the group was that there had not been a systematic approach to study the mechanism of action of anti-sense RNA or to investigate the construction of vectors used to express the anti-sense sequences in cells. It is planned to have the results of this discussion group presented at the October 1986 meeting of the DCE Board of Scientific Counselors and to propose an RFA targeted to these specific issues.

A great deal has been learned in the past decade about genes involved in cell transformation and the mechanism of their action. Most cancers in vivo and most in vitro transformation phenomena result from the expression of more than one gene. Many investigators have referred to known viral and cellular oncogenes as being in two classes: immortalizing or establishment genes in one class and transforming genes in a second class, with a member of each class being necessary to establish transformation of primary cells in vitro. Clearly, cell transformation is more complicated, since not all cells of all established cell lines become transformed by the expression of transforming genes. Some continuous cell lines can be transformed by immortalizing genes or transforming genes and some already established cell lines require expression of transforming genes and other establishment genes to complete their transformation. Even though many viral or cellular oncogenes are naturally active in a restricted set of differentiated tissues, in many instances, expression of the gene from heterologous promoters in other cells frequently results in the display of its immortalizing or transforming phenotype. The evolution of cancers in vivo may require expression of an even more complex repertoire of genes.

In summary, much progress has been made in elucidating the regions of the genomes of herpes- and adenoviruses involved in transformation in vitro. However, further studies must be done in order to elucidate the mechanisms by which these viruses actually cause oncogenesis in vivo. The final goal of such studies is the control and reversal of this process.

DNA VIRUS STUDIES I
GRANTS ACTIVE DURING FY86

<u>Investigator/Institution/Grant Number</u>	<u>Title</u>
1. AURELIAN, Laure University of Maryland (Baltimore) 5 R01 CA 39691-02	Transformation by Restriction Fragments of HSV DNA
2. BERK, Arnold J. University of California (Los Angeles) 5 R01 CA 25235-08	Biosynthesis of Adenovirus Early RNAs
3. BERK, Arnold J. University of California (Los Angeles) 5 R01 CA 41062-02	Transcription Stimulation by Adenovirus E1A Protein
4. BROWN, Nathaniel A. University of California (Los Angeles) 5 R01 CA 35536-03	Human Lymphocytes Clonally Transformed by EBV
5. CALNEK, Bruce W. Cornell University (Ithaca) 2 R01 CA 06709-24	Studies on the Avian Leukosis Complex
6. CHANG, Robert S. University of California (Davis) 1 R01 CA 43051-01	Epstein-Barr Virus and Nasopharyngeal Carcinoma
7. CHINNADURAI, Govindaswamy St. Louis University 5 R01 CA 31719-05	Genetic Analysis of Adenovirus 2 Early Genes
8. CHINNADURAI, Govindsawamy St. Louis University 2 R01 CA 33616-07	Adenovirus 1p Locus: Role in Oncogenic Transformation
9. CLOUGH, Wendy G. University of Southern California 5 R01 CA 23070-10	EBV DNA Synthesis in Transformed Lymphocytes
10. CLOUGH, Wendy G. University of Southern California 5 R01 CA 35343-03	DNA Methylation in Lymphocytes

11. COOK, James L.
National Jewish Hospital
and Research Center
5 R01 CA 38796-02
Tumor Cell Resistance to
Destruction by Effector Cells
12. COOPER, Neil R.
Scripps Clinic and
Research Foundation
2 R01 CA 14692-14
Humoral Immunity to Viruses and
Virus-Infected Cells
13. COURTNEY, Richard J.
Louisiana State University
Medical Center (Shreveport)
5 R01 CA 42460-02
Studies of Purified Herpes
Simplex Virus Glycoproteins
14. CROCE, Carlo M.
Wistar Institute of Anatomy
and Biology
5 R01 CA 16685-11
Mapping of Tumor Virus Genomes
in Transformed Cells
15. DE MARCHI, Jeanette M.
Vanderbilt University
5 R01 CA 20806-09
Induction by Cytomegalovirus
of Cell DNA Synthesis
16. DESROSIERS, Ronald C.
Harvard University
5 R01 CA 31363-05
Molecular Basis for Herpesvirus
Saimiri Oncogenicity
17. EGGERDING, Faye A.
University of California
(Los Angeles)
2 R01 CA 25545-04
Regulation of Adenovirus 2
Transcription
18. FIELDS, Bernard N.
Harvard Medical School
1 R13 AI/CA 22357-01
Gordon Conference on Animal
Cells and Viruses
19. GALLOWAY, Denise A.
Fred Hutchinson Cancer
Research Center
5 R01 CA 26001-08
Herpesvirus Expression in
Transformation and Latency
20. GALLOWAY, Denise A.
Fred Hutchinson Cancer
Research Center
5 R01 CA 35568-03
Molecular Studies on
Herpesvirus Proteins
21. GAYNOR, Richard B.
University of California
(Los Angeles)
5 R01 CA 30981-06
Transforming Functions of
Adenovirus E1A Proteins

22. GLASER, Ronald
Ohio State University
5 R01 CA 29066-06
Epstein-Barr Virus DNA in Transfected Cells
23. GLASER, Ronald
Ohio State University
5 R01 CA 36357-03
Molecular Genetics of Epstein-Barr Virus
24. GLASER, Ronald
Ohio State University
1 R13 CA 43451-01
International Symposium on EBV and Associated Diseases
25. GREEN, Maurice
St. Louis University
5 R01 CA 21824-10
Transforming Proteins of Three Human Adenovirus Groups
26. GREEN, Maurice
St. Louis University
5 R01 CA 29561-29
Biochemistry of Animal Virus Multiplication
27. HARDWICK, Jan Marie
Johns Hopkins University
1 R01 CA 43532-01
Epstein-Barr Virus: Regulation of Gene Functions
28. HARTER, Marian L.
University of Medicine and Dentistry of New Jersey (Newark)
5 R01 CA 28414-06
Function of Early Proteins Encoded by Adenovirus Type 2
29. HAYWARD, Gary S.
Johns Hopkins University
5 R01 CA 22130-09
Structure and Regulation of Human Herpesvirus Genomes
30. HAYWARD, Gary S.
Johns Hopkins University
5 R01 CA 28473-06
Cellular Transformation by DNA of Human Herpesvirus
31. HAYWARD, S. Diane
Johns Hopkins University
5 R01 CA 30356-05
EBV Genome Expression: Localization of Specific Functions
32. HAYWARD, S. Diane
Johns Hopkins University
1 R01 CA 42245-01
Regulation of Replication and Latency by EBV EBNA1
33. HENLE, Werner
Children's Hospital of Philadelphia
5 R01 CA 33324-03
EBV Serology in Human Cancers and Immune Deficiencies

34. HIRSCH, Martin S.
Massachusetts General Hospital
5 R01 CA 12464-16
Immune Reactivity and Oncogenic
Virus Infections
35. HORWITZ, Marshall S.
Yeshiva University
5 R01 CA 11512-17
Adenovirus DNA Synthesis and
Polypeptide Assembly
36. HOWETT, Mary K.
Pennsylvania State University
Hershey Medical Center
5 R01 CA 25305-08
Modulation of the Tumorigenicity
of Transformed Cells
37. HUANG, Eng-Shang
University of North Carolina
(Chapel Hill)
5 R01 CA 21773-08
Cytomegaloviruses and Human
Malignancy
38. HYMAN, Richard W.
Pennsylvania State University
Hershey Medical Center
2 R01 CA 16498-12
Malignancy and DNA Homology
among the Herpesviruses
39. ISOM, Harriet C.
Pennsylvania State University
Hershey Medical Center
5 R01 CA 23931-09
Regulation of Differentiation
in Hepatocytes in Vitro
40. KIEFF, Elliott D.
University of Chicago
5 R01 CA 17281-12
EBV Interaction with
Lymphoblasts in Vitro and
in Vivo
41. KLEIN, George
Karolinska Institutet
5 R01 CA 28380-05
EBNA and Other Viral Products
in EBV Transformed Cells
42. KLEIN, George
Karolinska Institutet
5 R01 CA 30264-05
Immune Effector Mechanisms in
EBV-Carrying Patients
43. KNIPE, David M.
Harvard University
5 R01 CA 26345-07
Genetics of Herpesvirus
Transformation
44. LEVINE, Myron
University of Michigan
(Ann Arbor)
1 R13 CA 40850-01
Tenth International Herpesvirus
Workshop

45. LEWIS, James B.
Fred Hutchinson Cancer
Research Center
5 R01 CA 29600-05
Functions of Adenovirus
Proteins in Transformation
46. LEWIS, James B.
Fred Hutchinson Cancer
Research Center
1 R01 CA 39636-01
Adenoviral Oncogene Expression
and Transformation
47. MATHEWS, Michael B.
Cold Spring Harbor Laboratory
5 P01 CA 13106-15
Cold Spring Harbor Laboratory
Cancer Research Center
48. MC DOUGALL, James K.
Fred Hutchinson Cancer
Research Center
5 R01 CA 29350-06
The Biology of Transformation
by Herpesvirus
49. MEDVECZKY, Peter G.
University of Massachusetts
(Worcester)
1 R01 CA 43264-01
Growth Factors and Herpesvirus
Saimiri Induced Lymphoma
50. MILLER, I. George, Jr.
Yale University
5 R01 CA 12055-15
Studies of Epstein-Barr Virus
51. MILLETTE, Robert L.
Portland State University
5 R01 CA 39067-03
In Vitro Transcription of Herpes
Simplex Virus Genes
52. MUNNS, Theodore W.
Washington University
5 R01 CA 27801-07
Immunochemical Characterization
of Nucleic Acids
53. NEMEROW, Glen R.
Scripps Clinic and Research
Foundation
5 R01 CA 36204-02
Infection of B Lymphocytes by
Epstein-Barr Virus
54. NONOYAMA, Meihan
Showa University Research
Institute for Biomedicine
in Florida
5 R01 CA 31949-06
Marek's Disease Virus:
Transformation and Oncogenesis
55. NONOYAMA, Meihan
Showa University Research
Institute for Biomedicine
in Florida
5 R01 CA 31950-04
Oncogenicity of Epstein-Barr
Virus

56. NONOYAMA, Meihan
Showa University Research
Institute for Biomedicine
in Florida
5 R01 CA 36895-02
Latency of Epstein-Barr Virus
DNA in Transformed Cells
57. PADMANABHAN, Radha K.
University of Kansas
Medical Center
5 R01 CA 33099-03
Structure and Functional
Analysis of Adenovirus Genomes
58. PAGANO, Joseph S.
University of North Carolina
(Chapel Hill)
5 P01 CA 19014-10
DNA Virus Genomes, Oncogenesis
and Latency
59. PEARSON, Gary R.
Georgetown University
5 R01 CA 39617-03
Epstein-Barr Virus-Induced
Membrane Antigens
60. PEARSON, George D.
Oregon State University
2 R01 CA 17699-11
Replication of an Oncogenic
Virus
61. PRUSOFF, William H.
Yale University
5 R01 CA 05262-26
Iododeoxyuridine, Iodo-DNA
and Biological Activity
62. RAAB-TRAUB, Nancy J.
University of North Carolina
(Chapel Hill)
5 R01 CA 32979-03
EBV Transcription in
Nasopharyngeal Carcinoma
63. RAPP, Fred
Pennsylvania State University
Hershey Medical Center
5 P01 CA 27503-07
Herpesviruses and Neoplasia
64. RAPP, Fred
Pennsylvania State University
Hershey Medical Center
2 R01 CA 34479-04
Latency and Transformation
by Herpesviruses
65. RASKA, Karel, Jr.
University of Medicine and
Dentistry of New Jersey
Rutgers Medical School
2 R01 CA 21196-09
Adenovirus T-Surface Antigens
and Tumorigenicity

66. REKOSH, David M.
State University of New York
(Buffalo)
5 R01 CA 25674-08
Adenovirus Early Gene Function
and DNA Replication
67. RICCIARDI, Robert P.
Wistar Institute of Anatomy
and Biology
5 R01 CA 29797-05
Organization and Expression of
Adenovirus Genes
68. ROBERTS, Bryan E.
Harvard University
5 R01 CA 27447-08
Organization and Expression of
Genes in Viral DNAs
69. ROBINSON, Robin A.
University of Texas Health
Science Center (Dallas)
5 R23 CA 36143-03
Regulation of Cellular Gene
Expression by HSV ICP-4
70. ROEDER, Robert G.
Rockefeller University
5 R01 CA 34223-04
Regulation of Adenovirus
Transcription
71. ROIZMAN, Bernard
University of Chicago
2 R37 CA 08494-21
Mechanisms of Viral Infection
in Relation to Cancer
72. ROIZMAN, Bernard
University of Chicago
5 P01 CA 19264-11
UCCRC: Viral Oncology Program
73. SCHAFFER, Priscilla A.
Dana-Farber Cancer Institute
5 R01 CA 20260-10
Immediate-Early Genes of HSV
74. SCHNEIDER, Robert J.
New York University
Medical Center
1 R01 CA 42357-01
Translational Regulation of
Adenovirus Gene Expression
75. SHARP, Phillip A.
Massachusetts Institute
of Technology
5 R01 CA 38660-02
Transcription Regulation by
Oncogenes
76. SHENK, Thomas E.
Princeton University
5 R01 CA 38965-03
Structure and Function of DNA
Tumor Virus Genomes
77. SILVERSTEIN, Saul J.
Columbia University (New York)
5 R01 CA 17477-12
Molecular Biology of Herpes-
virus

78. SIXBEY, John W.
St. Jude Children's
Research Hospital
5 R23 CA 38877-02
Epstein-Barr Virus Expression
in Normal Human Epithelium
79. SPEAR, Patricia G.
University of Chicago
5 R01 CA 21776-10
Herpesvirus Gene Expression in
Transformed Cells
80. SPECK, Samuel H.
Dana-Farber Cancer Institute
1 R01 CA 43143-01
Viral Transcription in EBV
Transformed Human B Cells
81. SPECTOR, David J.
Pennsylvania State University
Hershey Medical Center
5 R01 CA 34381-03
Regulation of Adenovirus
E1B Gene Expression
82. SPECTOR, Deborah H.
University of California
(San Diego)
2 R01 CA 34729-04
Human CMV, Cell-Related DNA,
Oncogenes and Kaposi's Sarcoma
83. SPELSBERG, Thomas C.
Mayo Foundation
5 R01 CA 25340-06
A New Class of Epstein-Barr
Virus Nuclear Antigen
84. ST. JEOR, Stephen C.
University of Nevada (Reno)
5 R01 CA 28089-06
Herpesvirus Induced Malignancy
85. STROMINGER, Jack L.
Dana-Farber Cancer Institute
5 P01 CA 21082-10
Molecular Basis of Viral
Oncogenesis
86. SULLIVAN, John L.
University of Massachusetts
(Worcester)
1 R01 CA 39653-01
Lymphotropic Herpesvirus of
Cottontail Rabbits
87. SUMMERS, William C.
Yale University
5 R01 CA 13515-13
Genetic Study of Animal Viruses
88. THORLEY-LAWSON, David A.
Tufts University
5 R01 CA 31893-05
Epstein-Barr Virus Membrane
Antigen
89. TIBBETTS, Clark J.
Vanderbilt University
5 R01 CA 34126-04
Adenovirus Genome Expression/
Assembly/Transfection

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| 90. WAGNER, Edward K.
University of California
(Irvine)
5 R01 CA 11861-17 | Control of Viral RNA Synthesis
in Herpesvirus Infection |
| 91. WENTZ, William B.
Case Western Reserve University
5 R01 CA 31973-03 | Sexually Transmitted Disease
in Uterine Carcinogenesis |
| 92. WILLIAMS, James F.
Carnegie-Mellon University
5 R01 CA 21375-09 | Genetic Analysis of Adenoviruses |
| 93. WILLIAMS, James F.
Carnegie-Mellon University
5 R01 CA 32940-05 | Type 12 Adenovirus
Transformation-Defective Mutants |
| 94. WOLD, William S.
St Louis University
2 R01 CA 24710-08 | Adenovirus 2 Coded Early
Glycoprotein |
| 95. WOLD, William S. M.
St. Louis University
2 R01 CA 33101-05 | Regulatory Features of HSV
Gene Expression |
| 96. YATES, John L.
New York State
Department of Health
(Buffalo)
1 R01 CA 43122-01 | The Functions of Epstein-Barr
Virus Nuclear Antigen I |

CONTRACTS ACTIVE DURING FY86

<u>Investigator/Institution/Contract Number</u>	<u>Title</u>
97. NEEL, H. Bryan, III Mayo Foundation N01-CP-91006	Application of Epstein-Barr Virus Markers to Diagnosis and Prognosis of Nasopharyngeal Carcinoma and Occult Tumors of Nasopharynx Area in USA

SUMMARY REPORT

DNA VIRUS STUDIES II

The DNA Virus Studies II component of the Branch involves the investigation of the two major classes of mammalian small DNA tumor viruses: papillomaviruses and polyomaviruses. In the component, there are 94 research grants with an estimated total funding of 16.52 million dollars. These include traditional research grants, program project grants, conference grants, outstanding investigator grants and new investigator grants. The major research emphasis of this program component is the elucidation of the molecular events leading to the initiation and maintenance of cellular transformation by the small DNA tumor viruses and the elucidation of the role of these viruses in the etiology of human cancers. In terms of scientific areas, 51% of the grants deal with the structure and expression of viral genes, 25% deal with the biochemical properties and mechanisms of action of viral gene products, 14% deal with the expression and function of cellular genes that are involved in the transformation process, 7% deal with the potential etiological relationship of small DNA viruses to human cancers, and 3% deal with basic biochemical and physiological studies which use the small DNA tumor viruses as model systems. In terms of the viruses being studied, 45% of the grants involve the simian polyomavirus, SV40; 25% deal with viral model systems and related systems such as viral oncogenes; 15% concern human or animal papillomaviruses; 13% relate to mouse polyomavirus; and 2% deal with human polyomaviruses. Representative studies for these classes of viruses are described below.

Among the more notable advances in the DNA Virus Studies II program this year has been the continued progress in understanding the possible etiological relationship between human papillomaviruses (HPVs) and anogenital cancers including cervical carcinoma. Previously, investigators have identified and classified HPV DNA from many pre-malignant, malignant and metastatic lesions in humans. HPV types 16 and 18 were usually found associated with severe dysplasias and carcinoma in situ, whereas HPV-6 and 11 were associated with milder lesions. This year, two new genital papillomaviruses, HPV-31 and 35, have been identified. They are associated with more severe anogenital lesions. In addition, new in vitro transformation assays and DNA cloning methods have been used to continue the analysis at the molecular level of the transformation process (47).

Two major barriers to research on HPVs have been the lack of good model systems to study transformation and an inability to grow HPVs in the laboratory. During the past year, an investigator sponsored by this Branch component has applied a novel strategy to produce a mouse/human model which will grow virus, readily produces benign tumor, and may provide the basis of a true transformation assay. The model uses normal human epidermal tissues (cervical, laryngeal, skin, or neonatal foreskin) which are exposed in vitro to a human condyloma (genital wart) extract containing HPV-11. Small segments of these tissues are then placed under the renal capsule of nude mice. In the mice, the tissue exposed to condyloma extract developed into tissue masses with all the histological and biochemical characteristics of a human condyloma, a benign tumor. All epidermal tissues tested in the mouse/human model showed evidence of this benign transformation, although there was a clear tropism for anogenital tissues. Interestingly, human neonatal foreskin produced the most extensive growths which

practically effaced the mouse kidney. HPV-11 has been extracted from these transformed foreskin grafts. Sufficient virus was obtained to infect a new generation of normal neonatal foreskins. Thus, it will now be possible to produce HPV-11 and possibly other HPVs in the laboratory, ensuring an adequate supply for experimental purposes. These results demonstrate, for the first time, that an HPV can transform human tissue to a condylomatous state under laboratory conditions. They also demonstrate, for the first time, the replication of an HPV under laboratory conditions. These studies make it possible to investigate the mechanism of cellular transformation and replication of HPVs in a laboratory system that is free of the ethical restraints that limit such studies in patients. Studies are now underway to extend this model to attempt to produce malignant transformation by using both serial passages of infected tissues and treatment of the cells with various chemical and viral cocarcinogens (45).

The molecular characterization of the HPV transformation process is also proceeding by the use of recombinant DNA technology. Animal models such as bovine papillomaviruses and the Shope rabbit papillomavirus are being used and have provided important information applicable to the human viruses. In the bovine system, the gene product of the E6 open reading frame has been identified as a major transforming protein. The main thrust this year for HPV molecular research has been the characterization of the viral RNA transcripts and proteins of HPVs. Studies using HPV-1 and HPV-6 DNA as templates have demonstrated that they are transcribed and properly spliced in model systems. Most of the transcripts (90%) appear to be for the early protein (non capsid) region of the HPV genome. These are hypothesized to be the transformation-related proteins. Studies on HPV-16 DNA containing cells have identified RNA transcripts for the E6 and E7 open reading frames in both established transformed human cell lines and in tissues from cervical carcinomas. The E7 transcripts were the most abundant in these studies, leading the investigators to prepare antibodies against the E7 gene product. Using these antibodies, an E7 protein was detected in cellular extracts. These results suggest that the E7 and possibly the E6 gene products of HPV 16 may be transforming proteins. Their persistence in established transformed cells over many generations tends to support a role for E7 and/or E6 proteins in the maintenance of the transformed state. It is expected that more HPV proteins will be identified and characterized in the near future (18,91).

HPV-6 and its subtypes (6a-6d) are associated with mild anogenital dysplasias and are usually thought to be of low oncogenic potential. Recent work by investigators supported by this Branch component have indicated that subtype HPV-6b can undergo mutation to an apparently highly oncogenic variant. An unusual HPV was isolated from a highly aggressive verrucous carcinoma of the vulva which had progressed rapidly over a period of months rather than years and which recurred after successive surgical excisions. The viral DNA (HPV-vc) was molecularly cloned and found to have a high degree of homology to HPV-6b DNA. Comparison of the HPV-6b and HPV-vc DNA sequences demonstrated that an additional 106 bases were present in the HPV-vc genome. These additional nucleotides were located in the non-coding region of the viral genome which contains the putative viral DNA replication and early gene transcriptional control elements. The extra bases also contained homologies to various human genes, such as cardiac actin and interferon. Prior to the onset of her disease, the patient had had a 16 year history of HPV associated genital warts. The

variant virus found in this patient may represent an example of an insertional mutagenesis of HPV-6b which led to the breakdown of the controls that had maintained this subtype's relatively benign character. Continued studies of this and other such variants may reveal the steps that lead from latent HPV infection to neoplasia in humans (47).

Investigators have recently demonstrated the presence of HPV in tumors at sites other than the usual anogenital sites. One group of investigators performed an analysis of 217 tumors and normal tissues for HPV DNA. They found that 2% (4) of the samples did contain HPV DNA, and most of these were HPV-16 related sequences. The four positive tumors included carcinomas from the lung, intestine, and two from the tongue. Other investigators in Germany have performed similar types of tumor surveys and have confirmed the above results by finding an 8% positive rate for HPV DNA in tumors. It is also well established that laryngeal papillomas (which have a 15-30% conversion rate to carcinomas upon irradiation) are caused by HPV-6. Although technically benign, these laryngeal papillomas grow aggressively, are locally highly invasive and may cause death by blocking of the airway. These results indicate that a small, but significant, portion of human cancers of the oral cavity, lung and intestine may be of papillomavirus origin (28,56).

Simian virus 40 (SV40) has been a major focus of research for many of the projects supported by this Branch component. This small DNA tumor virus was isolated from monkeys and has long been used as a model system to study viral transformation of cells in culture and tumorigenesis in susceptible rodents. Previous research demonstrated that the transformation properties of SV40 are associated with the viral gene that encodes the large T-antigen. This protein dominates both the lytic and transforming interactions of SV40 with the cells it invades. It provides many biochemical functions for viral infection, including modulation of DNA transcription and replication and the production of cell surface antigens. In contrast to a growing number of viral oncogenes that require multiple proteins acting cooperatively to produce a fully transformed phenotype, the large T-antigen alone can convert primary cells into tumorigenic cells lines. All of the viral functions required to immortalize cells and to confer the capacity to grow well in low serum concentrations or to high saturation density, to grow as anchorage independent clones, or to grow as tumors in nude mice, reside in this protein. Thus, the large T-antigen gene and its protein product have become the primary area of investigation in SV40 mediated transformation studies.

Recent analyses of mutations in the large T-antigen gene have begun to better define the domains in the gene which correspond to specific biochemical properties of the protein. Study of these mutants indicates the DNA binding activity, but not ATPase activity, is affected by mutants with deletions in the vicinity of amino acid residue 138. In contrast, the T-antigens of mutants with deletions of amino acids near position 509 or 587 lack ATPase activity, but are able to bind to the SV40 origin of DNA replication. Mutants at all of these sites are able to provide adenovirus helper function and to complement SV40 mutants with deletions affecting the carboxyl terminus of large T-antigen. Thus, three functional domains can be defined: origin binding, ATPase activity, and adenovirus helper function. Interestingly, mutants lacking ATPase activity cannot complement mutants lacking origin binding activity, providing strong support for the suggestion that both functions of large T-antigen are needed for

initiation of viral DNA replication and that both must be present in a single large T-antigen monomer subunit. The ability of these mutants to transform primary mouse embryo fibroblast cells was also studied. The findings indicate that the carboxyl terminal 81 amino acid residues of large T-antigen are not required for full immortalization capacity. In contrast, removal of as few as three residues at position 587-589 severely restricts immortalization, suggesting that this region may be important for immortalization. Significant levels of immortalization were obtained with mutants defective for SV40 origin-DNA binding, ATPase activity, or adenovirus helper function, suggesting that immortalization and transformation depend upon still other (as yet undefined) properties of large T-antigen (19,81).

Analyses of T-antigen mutants defective in cell surface or membrane-associated functions of this protein are difficult to perform. However, the recent development of cloned cytotoxic T-lymphocytes (CTLs) specific for the membrane bound form of this protein allows a detailed study of the function of surface T-antigens. For example, it has been found that the amino terminal 127 and the carboxyl terminal 82 amino acids of large T-antigen are not needed for insertion into the cell membrane. The CTLs can also differentiate between T-antigens of SV40 and the human polyomavirus BK. Investigators have used these methods to develop a technique to select specific types of mutants of SV40. Cells newly transformed by SV40 are exposed to CTLs which will kill all transformed cells except mutants that are either incapable of expressing cell surface T-antigen or are missing the specific epitopes recognized by the CTLs. Such mutants are essential for correlating this property of the protein with its transforming function and immunogenicity in vivo (82,81,19).

The biochemical characterization of T-antigen protein has also progressed during the past year. The adenylation of T-antigen was reported in 1984 and at that time it was postulated that this activity was related to the initiation of DNA synthesis by T-antigens. Several additional pieces of evidence have accumulated that support this hypothesis. First, preliminary results suggest that active T-antigen may be covalently associated with a short RNA moiety. Short RNA segments can act as primers for DNA synthesis. In addition, T-antigen has recently been found to contain an internal thioester between cysteine 105 and glutamic acid 108. Such thioesters are seen in a number of cellular proteins and are associated with receptor recognition functions. Glutamic acid-residue 108 is also known to be necessary for the replication of SV40 DNA. When this cys 105-glu 108 thioester is broken by methylamine treatment, the resultant T-antigen can no longer be adenylated. However, the relationship between the thioester sensitivity to methylamine and adenylation must be indirect because the site of adenylation is most likely at serine residues 430 or 504 (52,12, 78,16).

Last year the Branch reported on the development of a new approach to study the tumorigenic activity of SV40 in vivo. Lines of transgenic mice were produced which carried the SV40 genome integrated into the mouse DNA and which transmitted these viral genes to their progeny in a Mendelian fashion. Several of these lines produce animals which predictably developed choroid plexus tumors of the brain. T-antigen was primarily expressed only in tumor tissue, which again demonstrates the central role of this protein in tumorigenesis. During the past year, these results have been significantly extended by both workers supported by this Branch and others. A series of deletion and substitution mutants have

been employed to demonstrate that the nucleotide sequences in the region of the SV40 enhancer (the 72 base pair repeat) is required to provide the tissue-specific expression of the SV40 large tumor antigen in transgenic mice. In addition, transgenic mice carrying an SV40 large T-antigen gene containing deletions failed to develop tumors. The kinetics of tumor formation and T-antigen expression in mice has been studied. By one to two weeks of age, SV40 large T-antigen can be detected in the brains of these mice. Since tumor growth and death do not occur in these mice until 15-16 weeks of age, it is possible that a second event is required to trigger tumorigenesis. Finally, mouse transgenic line 419 has been bred through six generations and out of 52 mice with SV40 DNA in all of their cells, only one mouse (at 1½ years of age) developed a tumor of the choroid plexus. Attempts to ascertain the difference between this mouse and other individuals of line 419 indicated that cytosine methylation patterns were altered in this mouse's tumor DNA compared to non-tumor tissue DNA in the same mouse or to other line 419 mouse tissues. The hypothesis that undermethylation of cytosine residues leads to SV40 T-antigen expression and subsequent tumor formation is being tested by administering azacytidine to these mice. Such studies should significantly increase our understanding of how activation of tissue specific proto-oncogenes, such as T-antigen, occurs (50).

The transformation mechanism of SV40 large T-antigen is mediated by cellular proteins which either interact with and/or are induced by this viral protein. The p53 cellular protein is the best known example of such a factor. It strongly binds to T-antigen and is also associated with tumors produced by other viral and chemical transformation agents. Studies using the recently isolated cDNA clone of this protein's gene have localized the probable T-antigen binding site to the region between amino acid residues 110 and 215 in the p53 sequence. Additional mutants of the p53 gene are being studied to determine the function of this protein in normal and transformed cells. Other cellular factors which are involved in SV40 transformation have also been identified during the past year. Most of these are cellular transcriptional factors such as SP1, LSF and CTF proteins which recognize both SV40 DNA sequences and homologous human DNA sequences (51,83,84,39).

Another, more unique, approach has been applied by one laboratory to identify cellular factors in SV40 transformation. This group has obtained a cDNA library of RNA transcripts which are overexpressed in transformed cells. Only a small number of cDNAs representing such genes were found. Two isolated cDNA clones represented mRNAs expressed at elevated levels in the transformed cell line in a manner relatively independent of growth conditions. The expression of three other cDNAs was dependent on the growth conditions of the cells, e.g., confluent or non-confluent growth. Interestingly, the only cDNA isolated that was expressed exclusively in the transformed cell was from an SV40 message. Analysis of one of the cellular cDNA clones has also provided evidence that SV40 transformation results in the activation of specific genes transcribed by the cellular RNA polymerase III. Characterization of these cDNAs and the proteins encoded by them is continuing (10).

A third virus whose study is supported by this Branch component is the mouse polyomavirus. It is similar to SV40 and its study has helped clarify the common features and possible variations in the transformation process by small DNA viruses. The SV40 and mouse polyoma virions are morphologically identical and

they contain nearly identical amounts of DNA. However, their genomes are organized differently, particularly with respect to the genes encoding the early tumor antigen region which are responsible for transformation. Polyoma DNA codes for six proteins including three tumor antigens: large T-antigen, middle T-antigen, and small T-antigen. The relationship among the tumor antigens with regard to cellular transformation is complex. The large T-antigen (which is localized in the nucleus) appears to be able to immortalize primary cells in culture, whereas middle T-antigen (which becomes membrane bound) is able to induce the transformed phenotype in previously immortalized cell lines. In the past year, investigators supported by this program have focused their studies on characterizing the structure and mechanism of action of these T-antigens. In particular, the intriguing 1983 observation that middle T-antigen forms a tight complex with the cellular protein pp60c-src has been vigorously pursued. This protein's association with middle T-antigen is the source of the latter's *in vivo* kinase activity. pp60c-src, a tyrosine phosphokinase, is the cellular homolog of the transforming protein encoded by the src gene of Rous sarcoma virus (RSV), i.e., pp60c-src is the product of a known cellular proto-oncogene.

Recent work on C-terminal mutants of mouse polyomavirus middle T-antigen (mT) has shed light on the mechanism of this protein's association with pp60c-src. A series of truncated mT proteins were produced. The mutant proteins failed to become membrane bound, had no associated kinase activity, and could not support transformation. Thus, membrane attachment of mT protein appears to require the 21 residue hydrophobic sequence at the carboxyl terminus and to be important for pp60c-src binding. Detailed examination of one mT mutant, Py-1387-T, has suggested a specific mechanism for this association. The mutant protein undergoes phosphorylation at only one of the two serine sites known to be phosphorylated in the cell, namely the 56 kilodalton (kD) specific site. The other site, the 58 kD specific serine site, though present in the mutant mT molecule, is not phosphorylated. Recent evidence has shown that protein kinase C, a cellular protein, is involved in the phosphorylation of mT *in vivo* to create the 58 kD form. The failure of the mutant mT to attach to membranes appears to prevent recognition of mT as a substrate by protein kinase C. This observation is consistent with current knowledge about the activation of this phospholipid-dependent calcium-activated kinase by membrane association. Previous work has also shown that "wild type" mT protein could be cleaved from membranes *in vitro*. These enzymatically derived fragments of mT are phosphorylated at the 58 kD site, but lacked the same amino acids as the mutant mTs. Yet, the fragment could still bind with and be phosphorylated by pp60c-src. Thus, it is the ability of mT to be phosphorylated at the plasma membrane rather than binding at the membrane or the retention of the C-terminal fragment per se that is important for mTs association with pp60c-src (7,8,26,68).

In addition to the cellular protein kinase C, investigators have also found a second cellular protein which apparently binds to the mT protein. Using antibodies specific for mT peptides, both pp60c-src and a previously undetected 61 kD protein co-purified with mT by immunoaffinity chromatography. A variety of mT mutants were then used to study the relationship between the associated proteins and the transforming ability of the mT-antigen. The mT-antigen of the nontransforming mutant, NG-59, failed to form a stable complex with either pp60c-src or the 61 kD protein, whereas mT-antigens of viruses competent for transformation formed complexes with both proteins. Interestingly, the majority of the 61 kD protein was in a complex with a different apparent molecular weight

(about 120 kD) from the complex containing the protein kinase activity (about 200 kD); therefore, the 61 kD protein may not be involved in the interactions between the mT antigen and pp60c-src. The results of the purification experiments using mutant mT antigens suggest that both pp60c-src and the 61 kD protein may be important for transformation (26,87).

The human polyomaviruses are a group of related viruses initially isolated from man. The main representatives of this group are the JC and BK viruses. They are structurally, genetically and immunologically related to SV40. These viruses produce latent infections in man and are capable of transforming human cells in culture and inducing tumors in experimental animals. Thus, there is interest in these viruses as possible human cancer viruses. On a basic research level, their study can provide insight into the mechanisms of human cell transformation.

The genetic similarity among the human polyomaviruses and SV40 has permitted the construction of hybrid viruses; e.g., JC-SV40 and JC-BK hybrids where the regulatory DNA sequences of one virus are attached to the T-antigen genes of the other virus. Examination of the JC virus regulatory sequences was of particular interest, since previous work has shown that the enhancer within this sequence confers a specificity to the JC virus for cells of neural origin for both lytic growth and transformation. Contrary to expectation, hybrids with a JC regulatory sequence, but with SV40 T-antigen genes, functioned efficiently in transformation assays in all cells tested. This suggests that the T-antigen, rather than the regulatory sequences, has the largest influence on JC-restricted transformation activity. Lytic studies also yielded significant results. Hybrid DNAs containing the JC regulatory region were lytically active in primary human fetal glial cells. This is the first example of such a radical alteration of a papovaviral genome producing viable virus. The JC virus regulatory machinery can initiate proper transcription of SV40 and BK virus early mRNAs. The SV40 and BK proteins subsequently made can interact productively with the JCV DNA sequences to initiate viral DNA replication and modulate early and late viral transcripts (31).

An additional accomplishment this year in the study of human polyomaviruses has been the derivation of permissive cell lines which allow the growth of JC virus. Previously, stocks of this virus could only be grown in primary human fetal cells which are very difficult to obtain. The new cell lines were produced by transforming primary human fetal glial cells with replication defective JC mutants. The resultant cell lines have integrated JC genomes, and constitutively produce JC T-antigens. Thus, these cell lines are similar to COS cell lines containing the SV40 genome. Besides providing adequate stocks of JC virus, it is expected that these cell lines will also be useful for studies on regulatory and T-antigen mutants of JC virus (31).

A workshop on "The Transformation Mechanisms of Papillomaviruses" was sponsored by this Branch on February 18-19, 1986. The goals of this workshop were to assess the state of the art in the field of papillomaviruses and to determine whether particular areas of study within the field should be stimulated. The recommendations of the workshop participants are currently being evaluated for possible program initiatives for presentation to the DCE Board of Scientific Counselors in FY87. Among the initiatives being considered are an RFA for

studies on Papillomavirus-Host Interactions and a Small Business Innovative Research (SBIR) announcement for the development of specialized monoclonal antibodies to HPV proteins for research.

A Request for Applications (RFA) entitled "The Role of Human Papillomaviruses in the Etiology of Cervical Cancer" was issued on February 1, 1985 with an application due date of June 1, 1985. The objectives of this RFA were to stimulate basic and clinical research on the role of primary HPV infections of the cervix in the progression of cervical dysplasias to carcinomas. Seventeen applications were received and they were reviewed for technical merit on December 1, 1985 by a special peer review committee. Seven applications resulting from this RFA were funded during FY86.

A second RFA entitled "The Transformation Mechanisms of Human Polyomaviruses" was issued on January 29, 1986. This RFA was developed from the recommendations of a workshop sponsored by this Branch on "Oncogenic Human Polyomaviruses" which was held on March 7, 1985. The primary objectives of the RFA were to stimulate basic studies on the transformation mechanisms of human polyomaviruses and to ascertain their possible role in human malignancies. The application due date was July 15, 1986. Grants in response to this RFA receiving a fundable priority score are expected to be awarded in FY87.

In summary, during the past year, evidence has continued to accumulate for a role of human papillomaviruses in the etiology of anogenital and possibly other human cancers. A major advance this year has been the development of a new model system using HPV-infected human tissue grafted into an animal host. This model for the first time will permit the study of the condylomatous transformation of human cells by HPV and provide a source of laboratory grown HPV stocks. Studies using immunological and recombinant DNA technology have also identified an HPV protein, the E7 gene product, in transformed human cells, suggesting that this protein may be a transforming agent. In addition, work on the transforming proteins of SV40 and human and animal polyomaviruses has continued to elucidate the molecular steps in the transformation process. The cellular proteins, which interact with these viral transforming proteins or with viral DNA, are being studied to determine their role(s) in the transformation process.

DNA VIRUS STUDIES II
GRANTS ACTIVE DURING FY86

<u>Investigator/Institution/Grant Number</u>	<u>Title</u>
1. ALONI, Yosef Weizmann Institute of Science 5 R01 CA 14995-12	Control of Gene Expression in Tumor Viruses and Cells
2. ALWINE, James C. University of Pennsylvania 5 R01 CA 28379-06	Regulation of DNA Tumor Virus Gene Expression
3. ALWINE, James C. University of Pennsylvania 5 R01 CA 33656-04	Control of Late Gene Expression in DNA Tumor Viruses
4. BASILICO, Claudio New York University 5 R01 CA 11893-16	Cellular and Viral Control of Oncogenic Transformation
5. BASILICO, Claudio New York University 2 P01 CA 16239-12	Biosynthesis in Normal and Virus-Transformed Cells
6. BASILICO, Claudio New York University 1 R35 CA 42568-01	Viral and Cellular Gene Expression and Growth Regulation
7. BENJAMIN, Thomas L. Harvard University 2 R01 CA 19567-10	Mechanism of Cell Transformation by Polyomavirus
8. BENJAMIN, Thomas L. Harvard University 2 R01 CA 25390-08	Effects of HR-T Mutations on Polyoma Gene Expression
9. BERG, Paul Stanford University 5 R01 CA 31928-05	Transduction of Genetic Information Related to Cancer
10. BOTCHAN, Michael R. University of California (Berkeley) 5 R01 CA 30490-06	Transformation of Cells by SV40 Virus
11. BOTCHAN, Michael R. University of California (Berkeley) 1 R01 CA 42414-01	Bovine Papillomavirus - Model Systems

- | | |
|---|--|
| 12. BRADLEY, Margaret K.
Dana-Farber Cancer Institute
5 R23 CA 38069-03 | Nucleotide Binding Properties of
SV40 Large T Protein |
| 13. BUTEL, Janet S.
Baylor College of Medicine
2 R01 CA 22555-09 | Biological Properties of SV40
Early Proteins |
| 14. BUTEL, Janet S.
Baylor College of Medicine
5 R01 CA 25215-08 | Tumor Virus Effects on Mammary
Epithelial Cells |
| 15. CARMICHAEL, Gordon G.
University of Connecticut
Health Center
5 R01 CA 32325-03 | Regulation of Polyoma Early Gene
Expression |
| 16. CARROLL, Robert B.
New York University
5 R01 CA 20802-10 | Biochemical Properties of the
SV40 T-antigens |
| 17. CHEN, Suzie
Columbia University
5 R23 CA 36319-03 | Host Responses to In Vitro
Mutated SV40 |
| 18. CHOW, Louise T.
University of Rochester
5 R01 CA 36200-03 | Human Papillomavirus Gene
Expression |
| 19. COLE, Charles N.
Dartmouth College
5 R01 CA 39259-02 | The Molecular Biology of SV40
Large T-antigen |
| 20. CONRAD, Susan E.
Michigan State University
5 R01 CA 37144-03 | SV40-Induced Changes of Growth
Regulation in Host Cells |
| 21. CONSIGLI, Richard A.
Kansas State University
5 R01 CA 07139-23 | Studies in Polyoma Transformed
Cells: Virion Proteins |
| 22. DAWE, Clyde J.
Harvard University
5 R01 CA 38722-02 | Molecular Pathology of
Polyomavirus-Host Interactions |
| 23. DE PAMPILIS, Melvin L.
Harvard University
5 R01 CA 15579-13 | Tumor Virus DNA Replication:
A Probe Into Oncogenesis |
| 24. DI MAYORCA, Giampiero
University of Medicine
and Dentistry of New Jersey
5 R01 CA 25169-06 | BK Virus: A Human Papovavirus |

38. HALLICK, Lesley M.
Oregon Health Sciences University
5 R01 CA 24799-06
Psoralens as Probes for Viral
Nucleoprotein Structure
39. HANSEN, Ulla M.
Dana-Farber Cancer Institute
5 R01 CA 38038-02
Mechanism of Initiation at RNA
Polymerase II Promoters
40. HUNTER, Anthony R.
Salk Institute for Biological Studies
5 R01 CA 28458-06
Viral Transforming Proteins
41. IMPERIALE, Michael J.
University of Michigan (Ann Arbor)
5 R01 CA 19816-11
Role of SV40 Gene A in Cellular
Transformation
42. KELLY, Thomas J.
Johns Hopkins University
5 P01 CA 16519-12
Program on Molecular Biology of
Viral Tumorigenesis
43. KELLY, Thomas J.
Johns Hopkins University
5 R01 CA 40414-02
Replication of the SV40 Genome
44. KERN, Francis G.
New York University
1 R23 CA 41367-01
Factors Controlling Polyoma Gene
Expression
45. KREIDER, John W.
Pennsylvania State University
(Hershey)
1 R01 CA 42011-01
Human Papillomaviruses in
Cervical Cancer
46. LANCASTER, Wayne D.
Georgetown University
5 R01 Ca 32603-05
Role of Papillomavirus DNA in
Cell Transformation
47. LANCASTER, Wayne D.
Georgetown University
5 R01 CA 32638-05
Papillomavirus DNA and Antigens
in Cervical Neoplasia
48. LANFORD, Robert E.
Southwest Foundation
for Biomedical Research
5 R01 CA 39390-03
SV40 T-antigen: Model for
Nuclear Transport of Proteins
49. LEHMAN, John M.
Albany Medical College
of Union University
5 R01 CA 41608-02
Pathology of Neoplastic
Transformation

63. POLLACK, Robert F.
Columbia University
5 R01 CA 38883-02
Tumor DNA Transformation of
Diploid Cells: New Oncogenes
64. PRIVES, Carol L.
Columbia University
5 R01 CA 26905-07
Function/Expression of SV40 and
Polyoma Tumor Antigens
65. REEVES, William C.
Gorgas Memorial Institute
of Tropical Medicine, Inc.
1 R01 CA 42042-01
Human Papillomavirus and
Cervical Cancer in Panama
66. ROBERTS, Thomas M.
Dana-Farber Cancer Institute
5 R01 CA 30002-05
Isolation of Polyoma T-antigens
Synthesized in E. Coli
67. RUNDELL, Mary K.
Northwestern University
5 R01 CA 21327-09
Functions of SV40 Small
T-antigen and Cellular Proteins
68. SCHAFFHAUSEN, Brian S.
Tufts University
2 R01 CA 34722-04
Products of the Transforming
Genes of Polyomavirus
69. SHAH, Keerti V.
Johns Hopkins University
5 R01 CA 13478-15
Investigation of SV40-Related
Infections of Man
70. SHAH, Keerti V.
Johns Hopkins University
1 R01 CA 42074-01
Outcome of Papillomavirus
Infections of the Cervix
71. SHENK, Thomas E.
Princeton University
1 P01 CA 41086-01
Viral and Cellular Oncogenes:
Mechanism of Action
72. SHODELL, Michael J.
Cold Spring Harbor Laboratory
1 R13 CA 39358-01
Origins of Female Genital Cancer
73. SIMMONS, Daniel T.
University of Delaware
5 R01 CA 36118-02
Structure and Function of the
SV40 Tumor Antigen
74. SMITH, Alan E.
Integrated Genetics, Inc.
1 R01 CA 43186-01
Mutagenesis of Papovavirus
Transforming Proteins
75. SOMPAYRAC, Lauren M.
University of Colorado (Boulder)
5 R01 CA 34072-03
SV40 Deletion Mutants:
Oncogenic Proteins

76. STEINBERG, Mark L.
New York University
5 R01 CA 27869-06
Phenotypic Modulation of Human
Infected Keratinocytes
77. SYRJANEN, Kari J.
University of Kuopio
1 R01 CA 42010-01
Natural History of Cervical HPV
Infections
78. TACK, Lois C.
Salk Institute for Biological Studies
2 R01 CA 37081-04
SV40 T-antigen, Chromatin
Structure and Viral Function
79. TEGTMEYER, Peter J.
State University of New York
(Stony Brook)
5 R01 CA 18808-12
Tumor Virus SV40: Protein
Function and DNA Replication
80. TEGTMEYER, Peter J.
State University of New York
(Stony Brook)
2 P01 CA 28146-06
Tumor Virus-Host Interactions
81. TEVETHIA, Mary J.
Pennsylvania State University
(Hershey)
2 R01 CA 24694-09
Mutagenesis of Specific Regions
of the SV40 Genome
82. TEVETHIA, Satvir S.
Pennsylvania State University
(Hershey)
5 R01 CA 25000-09
Biology of SV40 Specific
Transplantation Antigen
83. TJIAN, Robert T.
University of California (Berkeley)
5 R01 CA 25417-08
The SV40 Tumor Antigen
84. TJIAN, Robert T.
University of California (Berkeley)
5 R01 CA 34724-04
Autoregulation of SV40 Early
Gene Expression
85. VARSHAVSKY, Alexander J.
Massachusetts Institute of Technology
5 R01 CA 30367-05
SV40 Virus Structure and
Replication
86. VILLARREAL, Luis P.
University of California (Irvine)
5 R01 CA 42004-02
Gene Expression of a Small DNA
Tumor Virus: SV40
87. WALTER, Gernot F.
University of California (San Diego)
5 R01 CA 36111-03
SV40 and Polyomavirus
Transforming Proteins

88. WATTS, Susan L.
University of North Carolina
(Chapel Hill)
5 R23 CA 41414-03
Expression of Human
Papillomaviruses in Cell Culture
89. WATTS, Susan L.
University of North Carolina
(Chapel Hill)
1 R01 CA 42085-01
Cervical Neoplasia: HPV
Epidemiology and Molecular
Biology
90. WEISSMAN, Sherman M.
Yale University
5 P01 CA 16038-13
Program on the Molecular Basis
of Viral Transformation
91. WETTSTEIN, Felix O.
University of California (Los Angeles)
5 R01 CA 18151-11
Analysis of the Shope Papilloma-
Carcinoma System
92. WETTSTEIN, Felix O.
University of California (Los Angeles)
1 R01 CA 42126-01
Human Papillomaviruses in
Genital Tract Dysplasias
93. WILSON, John H.
Baylor College of Medicine
2 R01 CA 15743-13
Pathways of Information Exchange
in Somatic Cells
94. WOODWORTH-GUTAI, Mary
Roswell Park Memorial Institute
5 R01 CA 28250-06
SV40 DNA Replication in Animal
Cells

SUMMARY REPORT

RNA VIRUS STUDIES I

The RNA Virus Studies I component of the Branch primarily involves studies of murine and primate tumor viruses and also includes projects on feline, bovine, and rat tumor viruses. In this program, extramural research is supported by several funding mechanisms: traditional research grants (R01), program project grants (P01), conference grants (R13), cooperative agreements (U01), and outstanding investigator awards (R35). The funding level for 113 grants was 18.3 million dollars. These grants involve studies in the murine (67%), primate (19%), feline (9%), bovine (3%), and rat (1%) model systems in the following areas: gene organization and expression including studies of oncogenes; virus-cell interactions; characterization of the biological activity of retroviruses; studies of the inhibition of viral replication; investigations of virus-induced cell transformation; and development of assays for retroviral vaccines.

Studies in the RNA Virus Studies I area are concerned with the elucidation of the molecular events associated with the conversion by viruses of normal cells to the malignant phenotype. The malignant phenotype is a stably inherited trait: tumor cells give rise to offspring which are tumor cells. This suggests that oncogenic transformation may be the consequence of genetic alterations. This is clearly the case for cells transformed by oncogenic viruses, where specific viral genes are responsible for the maintenance of the neoplastic state. The question arises as to the function and identity of the genes responsible for naturally occurring tumors and the type(s) of genetic rearrangements thought to result in the aberrant activation of these genes. The observation that cellular homologues of viral oncogenes, in many instances, appear to be responsible for the transfer of the transformed phenotype to fibroblasts in culture has spurred a search for the mechanisms by which these endogenous cellular genes may become altered to produce products with a potential for causing malignant transformation. The mechanisms responsible for the activation of cellular oncogenes may involve (1) local changes or mutations in genes involving base changes or small deletions which alter the functional properties of the gene product, (2) gross changes in the relative position of genes which may either involve translocations of structural gene information from one chromosomal location to another or the introduction of activators (such as viral long terminal repeats) adjacent to cellular genes such that the level of gene expression is enhanced, (3) gene amplification mechanisms which may increase the amounts of specific gene products, and (4) changes in the activity of oncogene promoters either by changing the base sequence itself or by altering genome structure in the vicinity of regulatory information (e.g., through changes in the pattern of methylation, the degree of supercoiling, or other aspects of chromatin structure).

Retroviruses first attracted widespread attention as oncogenic agents that replicate via DNA intermediates and involve integration of DNA copies of their genomes in the host chromosomes. Previous studies of these agents have demonstrated that they function as agents with varied pathological potential, dispersed through many species and transmitted by vertical as well as horizontal routes; as parasites well-adapted to host functions, thereby facilitating the orderly integration and expression of viral genomes; as intermediates in the relocation of DNA proviruses, which are structural homologues of the

transposable elements of other organisms; as mutagens equipped to interrupt or activate cellular genes; and as vectors able to transduce cellular genes and potentially act as agents of evolutionary change. No other class of animal viruses exhibits such profound intimacy with the genomes of their hosts. Thus information gathered concerning this relationship should increase our understanding of the transformation process.

Retroviruses were previously classified into two groups: those that contain oncogenes and those that do not. Members of the first group (acute transforming retroviruses or rapidly transforming retroviruses) induce neoplastic disease in infected animals within a few weeks after infection and cause rapid transformation of target cells in tissue culture. These viruses contain oncogenes (v-onc genes) that are derived from normal cellular genes, the proto-oncogenes, by recombination. Viruses of the second group (slowly transforming retroviruses) lack oncogenes, induce neoplastic disease in animals only after a long latent period (4-12 months), and do not cause transformation of tissue culture cells at a detectable frequency. A third class of retroviruses consists of viruses such as spleen focus-forming virus (SFFV) and mink cell focus-forming virus (MCF), which appear to be envelope gene recombinants. Although these viruses, in some cases, rapidly induce lesions in infected animals, they do not appear to carry an oncogene of the classic type (i.e., a cell-derived oncogene). Rather, by some unknown mechanism, sequences located within the env region appear to be responsible for their pathogenic properties. Finally, in the past two years, a fourth group of lymphocyte transforming retroviruses without oncogene sequences (T-cell lymphotropic viruses, HTLV) have been recognized. They apparently bring about cellular transformation through a novel trans-activation mechanism involving a trans-acting protein encoded by the pX region (now termed tat) of the viral genome. Extramural research involving all four types of viruses is being administered by the RNA Virus Studies I component of the Biological Carcinogenesis Branch.

Rhesus monkeys at the New England Regional Primate Research Center suffer from a fatal immunodeficiency disease similar to human AIDS. Investigators at the Center, in collaboration with others at Harvard University, have isolated a retrovirus from affected animals which is serologically related to the human AIDS virus, HTLV-III. This simian virus, designated simian T-cell lymphotropic virus III (STLV-III), is capable of transmitting the fatal immunodeficiency disease to other Rhesus monkeys. In one such study, four of six inoculated monkeys died within 160 days of experimental virus inoculation. This simian virus infection may provide important insights into the natural distribution and pathogenicity of the related human retroviruses. More importantly, the existence of this new AIDS-like animal model may facilitate the development and evaluation of vaccination and therapeutic approaches against the causative agent of human AIDS in a readily available primate (18,27,28).

Recent studies of Essex and associates have also shown that a virus immunologically closely related to STLV-III causes natural infections in wild caught African green monkeys in Africa, in the apparent absence of disease. Because these investigators felt that the human AIDS virus might have a simian origin, they tested sera from healthy humans in Senegal, West Africa and found that they were infected with a virus which is more closely related to the African green monkey virus (STLV-III ^{AGM}) than to any other virus of the HTLV/LAV family of viruses. They designated this virus as HTLV-IV. This unique model of silent

and persistent infection with a "nonpathogenic" member of the AIDS family of retroviruses, provides insights into the diverse biological interaction of this family of retroviruses with primate hosts, including humans, and is thus important in the study of human AIDS and the development of a vaccine against AIDS. In addition, these studies provide suggestive evidence on the possible African nonhuman primate origin and subsequent transmission to man and to rhesus monkeys, of the HTLV/LAV family of retroviruses associated with immunodeficiency syndromes.

These investigators also found that cells infected with HTLV-III virus contain two viral glycoproteins, gp160 and gp120, reactive with serum antibodies in HTLV-III seropositive healthy homosexuals. These glycoproteins, encoded by a 2.5 kilobase open reading frame located in the 3' end of the HTLV-III genome, represent the major species of HTLV-III encoded envelope gene products. Detection of antibodies to these glycoprotein antigens of HTLV-III virus appears to be the most sensitive procedure for serodiagnosis of AIDS and may also be valuable in developing vaccination approaches. An additional protein of HTLV-III/LAV with a molecular weight of 27 kD (p27) has recently been identified and shown to have a coding origin 3' to the *env* gene on the viral genome. The presence of antibodies to p27 in virus-exposed individuals indicates that this protein is antigenic in the natural host (27,28).

The human T-cell leukemia viruses (HTLV) are associated with T-cell malignancies in man and transform normal human T-cells *in vitro*. The mechanism of malignant transformation by HTLV is unknown, but appears to be distinct from that of other classes of retroviruses, which induce malignant transformation through viral or cellular oncogenes. Recently a new gene, the *tat* gene also termed x (originally designated pX), was identified in HTLV. This gene has been hypothesized to be the transforming gene of HTLV because of its conservation within the HTLV class of retroviruses. Studies of Chen have confirmed the observations of Essex and Haseltine that proteins encoded by the x gene of HTLV-I and HTLV-II (40 kD (HTLV-I) and 37 kD (HTLV-II)) are directly responsible for inducing cell transformation through trans-activation mechanisms. It was shown that the product of the x gene is encoded by a 2.1-kilobase messenger RNA derived by splicing of at least two introns. The complete primary structure of the x gene product has been determined. By *in vitro* mutagenesis of the x gene of the HTLV-II, Chen further demonstrated that the presence of a functional x gene product is necessary for efficient HTLV transcription. Therefore, his studies provide direct evidence for an important function of the x gene in HTLV replication. The functional analogies between the x gene and transcriptional regulatory genes of some DNA viruses suggest that these viruses may share similar mechanisms for cellular transformation (11,27,28,51).

Haseltine, in collaboration with Drs. Gallo and Wong-Staal at the NCI, demonstrated that the causative agent of human AIDS, the HTLV-III virus, has certain properties similar to other human lymphotropic viruses (HTLV-I and HTLV-II) and the bovine leukemia virus. The similarity concerns the mechanism of causing enhanced virus growth (and eventually the death or malignancy of host cells) through a trans-acting factor, presumably a protein, coded for by the viral genome and mediated through the long terminal repeats (LTR) of the virus. Transfection of the HTLV-III LTR fragment attached to a gene with recognized activity, the chloramphenicol acetyl transferase (CAT) gene, into various types of virus-infected and control cells, resulted in enhanced activity of the CAT

gene only in cells infected with HTLV-III virus, but not in cells infected with other viruses or in control cells. This HTLV-III LTR enhancing factor failed to trans-activate the LTRs of other viruses such as HTLV-I and bovine leukemia virus, which were similarly transfected into HTLV-III infected cells. These results clearly suggest that the trans-acting factor, coded for by the HTLV-III viral genome specifically trans-activates HTLV-III LTRs to bring about enhanced virus growth, and presumably also brings about host cell death by similar trans-activation of cellular genes involved in limiting cell replication. Transcriptional trans-activation of the LTR in infected cells, thus places HTLV-III virus in the retroviral family that includes HTLV-I, HTLV-II, and bovine leukemia virus, which are associated with neoplastic disease and which bring about similar autostimulation of their own growth and malignant behavior of infected host cells through trans-acting proteins (51).

Leukemic T-cell lines that are infected with HTLV-I or -II uniformly display large numbers of membrane receptors for interleukin-2 (IL-2) and some, if not all, of these cell lines produce IL-2. Further studies of Haseltine and Chen have suggested that the trans-acting protein encoded by the tat gene may play an important role in cell transformation. Convincing evidence that this may, indeed, be the case was recently obtained. It was found that introduction of the tat gene of HTLV-II into the Jurkat T-lymphoid cell line resulted in the induction of IL-2 receptors and IL-2 gene expression. Since IL-2 promotes T-cell growth and is involved in auto-stimulatory control of cell growth, the gene coding for the trans-activation factor gene apparently brings about T-cell overgrowth through the over-induction of IL-2 receptors and IL-2 gene expression (51).

Additional studies of Haseltine and Wong-Staal and Gallo (at the NCI) used gene splicing techniques to remove the tat gene to determine what effect this would have upon virus replication. These studies showed that HTLV-III stopped reproducing entirely when its tat gene was deleted and suggest that control of the AIDS retrovirus in infected people may be achieved through drugs that would inactivate this key virus gene or the protein it produces. In addition, an AIDS virus particle that is intact, except for the tat gene, could potentially be valuable as a vaccine, since the human immune system would presumably develop protective antibodies against natural infection with this nonreplicating, but antigenically intact virus (51).

Recent transfection studies have shown that the HTLV-III virus whose natural tropism is for human T-4 lymphocytes is able to replicate in fibroblast cells of other species such as monkeys, minks, and mice. The virus reproduced poorly in these heterologous host cells, especially in mouse cells, a fact that suggests that animal cells may be lacking factors that more susceptible human cells have or that human cells lack an inhibitor of the virus that animal cells possess. The slow growth in animal cells also suggests that the AIDS virus has adapted to humans and has probably been in the human population for some time (OBC component reference 19).

Despite the long established association of FeLV infection with immunodeficiency disease, it was not known until recently whether feline AIDS (FAIDS) was caused by a specific FeLV variant or, alternatively, was a consequence of FeLV infection coupled with either host genetic factors, concurrent infections, or other environmental stresses or cofactors. Hoover, in collaboration with

Mullins, has shown that a specific FeLV variant, designated FeLV-FAIDS, induces a fatal immunodeficiency syndrome in 100% of viremic specific-pathogen-free (SPF) cats after an inoculation period whose length is dependent on the age of the cat at time of virus inoculation. Experimental FAIDS, like the natural disease, is characterized by persistent viremia, progressive weight loss, severe lymphoid depletion (apparently preceded by lymphoid hyperplasia), chronic intractable diarrhea, and opportunistic infections. Furthermore, the onset of disease coincides with the appearance of a characteristic FeLV-FAIDS variant, often manifested as unintegrated viral DNA in the bone marrow of cats. The FAIDS variant genome persists as unintegrated DNA: an unusual retrovirus/host cell relationship paralleled only by other cytopathic retroviruses such as visna virus, the avian osteopetrosis virus, and the human AIDS retrovirus, HTLV-III (54,66).

The FeLV-FAIDS retrovirus is distinguished by a novel KpnI endonuclease cleavage site within the gp70 region of the viral genome. This feline retrovirus is recognized in two major forms in infected cats: a "common" form found in the asymptomatic phase of infection in all infected tissues of all infected cats and a "variant" form, which is disease-specific, appears to arise or preferentially replicate in the bone marrow and presages the clinical onset of irreversible feline AIDS. The feline AIDS-inducing virus, unlike the human AIDS virus, does not contain a long open reading frame region between the envelope gene and the 3' LTR; however, the FAIDS virus does contain a potential alternate open reading frame in the gag region spanning p15-p12 domains. Finally, and perhaps of greatest biologic relevance, the human and feline immunosuppressive retroviruses both cause a fatal immunodeficiency syndrome with closely analogous clinical and pathologic features; the feline virus will induce this disease in 100% of inoculated cats after a predictable and relatively short incubation period (40 to 158 days) in weanling outbred SPF cats. Thus, studies of this strain of FeLV and the induced feline AIDS disease are fundamentally important as a model for the human AIDS.

In addition to its role in the etiology of feline leukemias, FeLV of subgroup C (FeLV-C) is known to cause anemia in cats. The mechanisms that underlie this specific erythroid inhibition by FeLV-C are not known. Rojko provided evidence that in vitro exposure to FeLV-C (Sarma isolate) directly depressed the development of colony forming units-erythroid (CFU_E) and burst-forming units-erythroid (BFU_E) from healthy FeLV-naive adult cat marrow cells. Addition of the lymphoma-causing Rickard strain of FeLV (FeLV-R/AB), which contains subgroups A and B and causes minimal to moderate anemias in vivo, was associated with constant depression of CFU_E and sporadic depression of BFU_E in vitro. On the other hand, in vitro exposure to the Kawakami-Theilen strain of FeLV, a phenotypic mixture of subgroups A, B, and C which causes rapidly fatal anemias in neonatal kittens, inhibited development of both CFU_E and BFU_E colony formation from adult cat bone marrow cells. Suppression of CFU_E in vitro was correlated with the in vivo pathogenicity of the virus strain and with level of virus replication in vitro in bone marrow cells. Similar interactions between hemopoietic cells and infectious, subgroup-specific viruses probably determine the rapidity and impact of anemia development in vivo (82).

The genomes of highly oncogenic retroviruses, the sarcoma and acute leukemia viruses, contain host cell-derived specific genes responsible for oncogenicity, which, in many cases, have partially replaced viral sequences necessary for normal

viral replication; thus, these viruses are replication defective. For example, the genome of Moloney sarcoma virus includes a single gene (mos) which is responsible for cellular transformation, but is not involved in virus replication. Other sarcoma and acute leukemia viruses contain different transforming genes. These transforming genes of sarcoma and acute leukemia viruses are homologous to DNA sequences present in normal, uninfected cells, the c-onc genes. These normal cell homologues of viral transforming genes are highly conserved in vertebrate evolution and appear to represent normal cellular genes whose function is still largely unknown. Transcription of several of these genes has been detected in normal and neoplastic cells and, in some cases, normal cell proteins have been identified which are closely related to proteins encoded by the homologous viral transforming genes.

The highly oncogenic sarcoma and acute leukemia viruses thus appear to represent recombinants in which a transforming gene, derived from a homologous gene of normal cells, has been inserted into a retrovirus genome. The transforming genes of these viruses are expressed at high levels in virus-infected cells as a consequence of their association with viral transcriptional regulatory sequences. It is thus possible that transformation by these viruses is a consequence of abnormal expression of normal cell genes. Alternatively, transformation might result from structural differences between the viral and cellular proteins.

The retroviral oncogene, v-fms, was acquired by genetic recombination between an FeLV and proto-oncogene sequences (c-fms) of normal cat cells. Sherr recently identified the c-fms proto-oncogene product and showed that it is related, and is possibly identical, to the receptor for the mononuclear phagocyte colony stimulating factor, M-CSF-1 or CSF-1. The v-fms coded glycoprotein transforms cells by an as yet unknown mechanism. The fact that the transforming protein retains the ability to bind CSF-1 suggests that transformation could be due to the viral transduction of a competent receptor gene into fibroblasts that produce the hormone. Alternatively, transformation may depend on aberrant v-fms coded tyrosine kinase activity. While the murine c-fms gene product exhibits enhanced tyrosine kinase activity in the presence of CSF-1, the v-fms gene product exhibits constitutively higher levels of enzyme activity. Thus, two nonexclusive mechanisms, autocrine stimulation in the case of murine c-fms and deregulated receptor kinase in the case of feline v-fms could contribute aberrant signals for growth (89).

Three cellular ras genes have been identified in the human genome (ras^H, ras^K, and ras^H), and activated forms of each have been detected in a wide variety of tumor cell DNAs by transfection of tumor cell DNA into NIH 3T3 cells. In human tumors, ras activation has been found to be a consequence of single amino acid substitutions at either position 12, 13, or 61 of the ras p21 protein. Random mutagenesis studies have shown that amino acid substitutions at positions 59 and 63 can also activate ras transforming potential. In addition to activation by structural mutations, overexpression of the normal ras^H protein has also been shown to be sufficient to transform NIH 3T3 cells. Using site-directed mutagenesis, Cooper introduced mutations encoding 17 different amino acids at position 61 of the human ras^H gene. Fifteen of these substitutions increased ras^H transforming activity. The remaining two mutants, encoding proline and glutamic acid, displayed transforming activities similar to the normal gene. Overall, these mutants varied over 1000-fold in transforming potency. Increased

levels of p21 expression were required for transformation by weakly transforming mutants. The mutant proteins were unaltered in guanine nucleotide binding properties. However, all 17 different mutant proteins displayed equivalently reduced rates of GTP hydrolysis, 8- to 10-fold lower than the normal protein. There was no quantitative correlation between reduction in GTPase activity and transformation potency, indicating that reduced GTP hydrolysis does not correlate with ras transforming potential (15).

Simian sarcoma virus (SSV), is an acutely transforming retrovirus which carries the oncogene v-sis. Recently, the predicted amino acid sequence of the v-sis gene product, p28_{sis}, was shown to have extensive sequence homology with one polypeptide chain of platelet-derived growth factor, PDGF. The protein p28_{sis} is immunologically related to PDGF. The initial v-sis translation product is processed at the amino and carboxyl-termini to generate lower molecular weight proteins. Although these lower molecular weight proteins are recognized by antisera to native PDGF, it is not known which form of the v-sis gene product is responsible for transformation. Donoghue found that the protein encoded by the v-sis oncogene contains a signal sequence, derived from the envelope gene of the parental retrovirus, which is required for transformation. Removal of the proposed signal sequence was correlated with loss of biological activity. This activity was restored to inactive deletion mutants by fusion with the coding region for a heterologous signal sequence. Biological activity of v-sis was also abolished by either a small deletion within the coding region of the signal sequence or by a point mutation introduced by site-directed mutagenesis (20).

The single transforming protein encoded by Abelson murine leukemia virus is a fusion of sequence from the retroviral gag genes with the v-abl sequence. Baltimore found that deletion of most of the gag region from the transforming protein results in a virus capable of transforming fibroblasts, but no longer capable of transforming lymphoid cells. Assessment of the effect of smaller deletions in gag demonstrated that p15 gag sequences are responsible for this effect; in contrast, deletion of p12 sequences had no effect on lymphoid transformation. In transformed fibroblasts, p15-deleted and normal proteins had similar activities and subcellular localization. When the p15-deleted genome was introduced into previously transformed lymphoid lines, its protein product exhibited a marked instability. The tyrosine-specific autophosphorylation activity per cell was less than 1/20th that of the nondeleted protein. Although pulse-labeling showed that the p15-deleted protein was synthesized efficiently, immunoblotting demonstrated that its steady-state level was less than 1/10th that of the nondeleted Abelson protein. The specific instability of the p15-deleted protein in lymphoid cells explains the requirement of these sequences for lymphoid, but not fibroblast, transformation (4).

By using small 5' or 3' deletions, it was determined that only 1.2 kilobases (kb) at the 5' end of the 3.9-kb v-abl sequence in Abelson murine leukemia virus is required for fibroblast transformation. Insertions of four amino acids, generated by putting synthetic DNA linkers into various restriction enzyme cleavage sites, abolished transforming activity, indicating that much of the internal sequence of the minimum transforming region plays a critical role in the transformation process. This 5' 1.2 kb of v-abl encodes protein-tyrosine kinase activity when expressed in E. coli. Each of the mutations which caused a loss of transformation activity also resulted in a loss of protein-tyrosine kinase activity when expressed in E. coli. The minimum transforming region of

v-abl contains amino acid homology to other protein-tyrosine kinase oncogenes (4,83).

Recombinant DNA technology makes it possible to tailor-make a rapidly oncogenic retrovirus by linking transforming onc genes from one virus with replicative and structural genes originating from different retroviruses. Using this approach, investigators hope to determine the specific components of the viral genome related to the induction and enhancement of oncogenesis. Fan generated a Moloney murine leukemia virus (M-MuLV) recombinant carrying most of the viral onc gene, v-src, from Schmidt-Ruppin A avian sarcoma virus and all other genes of M-MuLV. The v-src sequences (lacking a portion of the 5' end of v-src) were inserted into the p30 region of the M-MuLV gag gene so that M-MuLV gag and v-src were in the same reading frame. Transfection of this chimeric clone, pMLV(src), into NIH 3T3 cells, which were constitutively producing M-MuLV gag and pol protein, resulted in the formation of foci of transformed cells. The infectious transforming virus recovered from these transformed cells was designated M-MuLV(src). M-MuLV(src)-transformed cells contained two novel proteins of 78 and 90 kD. The 78 kD protein, p78gag-src, contained both gag and src determinants, exhibited kinase activity in an immune kinase assay, and is probably a fusion of the murine Pr65gag and src. The 90 kD protein, which is of the appropriate size to be the murine glycoprotein gPr80gag fused to avian src, contained gag determinants as well as a V8 protease cleavage fragment typical of the carboxyl terminus of avian sarcoma virus pp60src. However, it could not be immunoprecipitated with an anti-v-src serum. M-MuLV(src)-transformed cells showed elevated levels of intracellular phosphotyrosine in proteins, although the elevation was less than that seen in cells transformed with wild-type v-src. M-MuLV and amphotropic murine leukemia virus pseudotypes of M-MuLV(src) were inoculated into newborn NIH Swiss mice. Inoculated mice developed solid tumors at the site of inoculation after 3 to 6 weeks, with most animals dying by 14 weeks. Histopathological analysis indicated that the solid tumors were mesenchymally derived fibrosarcomas that were both invasive and metastatic. These studies confirm the fact that the src oncogene functions in a similar way whether attached to avian or mammalian retroviral sequences (32,33).

All of the slowly transforming retroviruses that are so far implicated in AKR leukemogenesis appear to be lacking an identifiable "leuk" (onc) protein, although conceivably the envelope proteins may function as such. Because of the absence of a readily defined onc gene as compared to the acute transforming retroviruses (e.g. RSV, HaSV, MC29), other hypotheses not involving a viral gene product directly responsible for transformation have been proposed for the mechanism of leukemogenesis by these viruses. Weissman and his co-workers have argued that persistent antigenic challenge and infection by the appropriate thymotropic virus leads to uncontrolled proliferation of a receptive T lymphocyte. Work by J. Ihle and his co-workers at the Frederick Cancer Research Facility (FCRF) support a similar hypothesis; however, the researchers at FCRF have shown that virus infection is not a prerequisite for T lymphocyte proliferation and that lymphokines may play a role in uncontrolled proliferation. Another possible mechanism is one analogous to the production of bursal tumors by the avian leukosis virus (ALV), which appears to occur by insertion of the provirus adjacent to a cellular c-onc. The promoter-like sequences in the LTR function as an initiator for transcription of the downstream cellular c-onc gene. The cellular gene in this instance is the c-myc gene, which is the cellular counterpart of the transforming gene of avian MC29

virus. However, bursal tumors and MuLV lymphocytic leukemias involve different target cells; therefore, the normal cellular gene involved in T-cell lymphomas may not be c-myc but an as yet unidentified c-onc gene. Past studies of Yoshimura have shown that there is an amplified expression of murine leukemia proviruses and viral RNAs in thymic lymphomas which strongly implicates MuLV infection of AKR thymocytes in AKR leukemogenesis. Her present studies are testing a possible promoter insertion mechanism (insertional mutagenesis) potentially involving a cellular oncogene or a T-cell differentiation gene in the induction of AKR lymphomas (108,113).

Carcinogenesis in AKR mice by MuLV appears to be due not only to appearance of env recombinant viruses, but also due to activation of a cellular c-onc gene by this recombinant virus. Recent studies of O'Donnell have confirmed the possibility suggested above that T-cell lymphomas initiated in AKR mice by env recombinant mink cell focus-inducing MCF viruses involve proviral integration of this recombinant virus into the c-myc locus. The question of insertional mutagenesis in AKR lymphomas was explored in a system in which leukemia was induced synchronously by intrathymic inoculation of env recombinant virus in young AKR mice. This system permits flow-cytometric detection of early phenotypic alterations in thymocytes before overt thymoma or disseminated leukemia occurs. By analysis of new proviral-host junction fragments in thymus DNA blots, O'Donnell found that the earliest phenotypically altered population represents outgrowth of one or a very few clones of cells. Specific proviral integrations into the c-myc locus were detected in some of these early clones. In later-developing thymomas and frank leukemias, up to 65% of the neoplasms exhibited clonal proviral alterations of c-myc. Thus, it appears that, as with retrovirus-induced B-cell lymphomas of chickens, virus-induced T-cell leukemia in mice is associated with a high frequency of proviral insertion into the c-myc locus (71).

Mouse mammary tumor virus (MMTV) has been implicated as the causative agent in the high incidence of mammary tumors observed in some inbred strains of mice. MMTV is a nonacutely transforming virus in that it does not transform cells in tissue culture and causes tumors only after a latency period of several months. There is no evidence that the virus encodes an oncogene. Oncogenesis by the slowly transforming MMTV also appears to be similar to the avian leukosis insertional mutagenesis model discussed above. In a majority of virally induced mammary tumors that have been examined, MMTV proviral DNA has been found at one of two genetic loci designated int-1 and int-2. These loci are on different chromosomes, and the DNAs that comprise the loci do not cross-hybridize with each other or with known oncogenes. Many aspects of the experimental observations can be explained by a model based on insertion of a transcriptional enhancer element, the glucocorticoid responsive element (GRE) present in MMTV DNA, into the genetic loci int-1 and/or int-2. Glucocorticoid hormones selectively activate the rate of transcription of these proviral genes by a mechanism that requires functional glucocorticoid receptor proteins. Preliminary studies of Peterson have uncovered a negative regulatory element in the MMTV long terminal repeat (LTR) which decreases transcription from the MMTV promoter in the absence of glucocorticoids (75).

The AKR SL3-3 virus is a highly leukemogenic ecotropic murine leukemia virus (MuLV) that was originally isolated from a mixture of viruses produced by an AKR thymic leukemia cell line, SL3. Haseltine, Lenz and coworkers have shown that

the transcriptional enhancer element located in the U3 region of the SL3-3 viral genome is primarily responsible for the enhanced oncogenic potential of this virus compared to the AKV, a closely related endogenous AKR virus. In contrast, the development of spontaneous leukemia in AKR and other high leukemia strains of mice appears to depend upon the formation of recombinant viruses that contain non-AKV sequences in the envelope gene, env, as well as in the U3 region. Many of these recombinants are leukemogenic following inoculation into neonatal mice and express envelope glycoproteins that permit virus infection of both mouse and heterologous cells (such as mink fibroblasts). Viruses that exhibit this host range phenotype are referred to as polytropic, dualtropic or mink cytopathic focus-forming viruses (MCFs). Despite the known association of viruses with recombinant envelope genes with spontaneous murine lymphomas, the relationship of such viruses to the induction of lymphomas by the ecotropic SL3-3 virus has not been well studied (50).

Thomas found that the SL3-3 virus readily induced or accelerated the onset of leukemia in mice of both high and low leukemia inbred strains. T₁-oligonucleotide fingerprints of viral RNAs and Southern blot analysis of proviral DNAs demonstrated that recombinant viruses were associated with tumor tissues from 14 of the 16 leukemic mice studied. These viruses appeared to be derived by recombination between the SL3-3 viral genome and endogenous viral sequences related to those found in the gp70 genes of polytropic MuLVs. These observations suggested that biological functions encoded in the polytropic virus-related env sequences of the recombinants contributed to the specific association of these viruses with the leukemic tissues. These functions are probably distinct from those encoded in the U3 region of the genome and may be similar to those mediated by the envelope genes of recombinant MuLVs previously recovered from spontaneous or other virus-induced murine leukemias (96).

Famulari is focusing on the identification of subgenomic regions of MCF MuLV required for thymic leukemogenesis. She previously determined that MCF virus-specific sequences in the U3 region of the LTR confer thymotropism on MuLV constructed in vitro. She further ascertained that the carboxyl terminal cytoplasmic tail of p15(E) affects the ability of this molecule to be transported to the plasma membrane of thymocytes. Specific amino acid substitutions found in MCF 247 virus relative to the ecotropic virus AKV appear to allow the transport and processing of the env gene polyprotein encoded by the pathogenic MCF MuLV. Studies are continuing to dissect the function of p15(E) in virus replication and pathogenicity in thymocytes in collaboration with Dr. Christie Holland (31,53).

Retroviruses that cause acute oncogenesis are generally complexes of a replication-competent helper virus and a replication-defective component. However, the pure defective components have not been previously available. Kabat prepared the defective spleen focus-forming virus (SFFV) component of Rauscher erythroleukemia virus (R-SFFV) by transfecting a colinear R-SFFV DNA clone into a retroviral packaging cell line. The transfected cells released virus that was free of helper virus and that induced erythropoietin-dependent erythroid burst formation in bone marrow cultures. When injected into normal adult NIH/Swiss mice in moderate doses, the helper-free SFFV caused a rapid splenic erythroblastosis that subsequently regressed. Extensive erythroblastosis could be maintained by repeated injections of helper-free SFFV into anemic mice or by the addition of a helper virus. It was concluded that R-SFFV, alone, causes

proliferation, but not immortalization of a population of erythroblasts that is normally replenished from a precursor stem cell pool. Because these precursor cells are inefficiently infected, a single, moderate inoculum of helper-free SFFV causes a wave of erythroblastosis. The properties of the proliferating erythroblasts are substantially determined by the R-SFFV viral component (59).

Gene transfer into the mouse germ line is a crucial test for cell-specific gene expression. Transgenic mice generated by pronuclear DNA injection have revealed abundant information about sequences that direct tissue-specific expression. However, such animals harbor mostly aberrant tandem organizations of the injected gene. These might affect neighboring chromatin structures, disrupt transcriptional units, and make quantitative studies on gene expression difficult to interpret. Therefore, the development of techniques to insert a single copy of a gene is of great importance. Retroviral infection mediates insertion of a single copy of a nonpermuted provirus per chromosomal site. Retroviral expression can be modulated by the enhancer sequences in the viral LTR, which determines tissue tropism and the type of disease caused by a particular MuLV. Until recently, only the Moloney strain of MuLV (M-MuLV), a highly leukemogenic replication-competent retrovirus, has been successfully inserted into the mouse germ line. Verma and collaborators have developed a general strategy for the insertion of both replication-competent and defective retroviruses into preimplantation mouse embryos, giving rise to transgenic mice that transmit the proviral DNA to offspring. Transgenic mice were generated that harbor a replication-competent recombinant retrovirus that lacks the Moloney murine leukemia virus (M-MuLV)-type enhancer sequence in the LTR. Instead, the LTR contains an enhancer element that permits polyoma virus F101 to grow in undifferentiated F9 embryonal carcinoma cells. Expression studies in different tissues of mice transgenic for recombinant M-MuLV indicate the possibilities to target and modulate expression of retroviral recombinants in mice by their LTR enhancer sequences (100,101).

Investigation of the roles of specific oncogene products in leukemogenesis, particularly in the T-cell lineage, continues to be the main focus of research of Fleissner. To study the physiological effect of altered myc regulation, he used two types of retroviral constructions in which mouse c-myc coding sequences are transcribed under the control of a viral enhancer. Insertion of a retroviral c-myc vector into fibroblasts either in the presence or absence of an activated ras gene has demonstrated that, for growth under anchorage-independent conditions, sensitivity to various growth factors is controlled by the myc gene product. Sources of growth factors used in these experiments have been either medium conditioned by the fibroblasts themselves or preparations of purified factors. Among the latter, transforming growth factor (TGF) has been found to be especially effective for cells expressing myc. Using a different vector construction, he is now extending these studies to cultures of thymocytes as well as other factor-dependent hemopoietic cell populations maintained in vitro. Preliminary results indicated that constitutive myc expression causes a sharp increase in sensitivity to interleukin 3 in one system. In a joint study with Dr. Louis Zeitz, Fleissner has found that introduction of a retroviral myc vector, or an activated ras gene into mouse fibroblasts increases the frequency of radiation-induced transformation. They are interested in pursuing this system as an in vivo model for radiation-induced neoplasias in vivo (36).

Fractionated x-irradiation of young adult C57/B1/6 mice induces T-cell lymphomas in greater than 95% of the treated mice in approximately 120 days (Kaplan and Brown, 1952). The mechanism of induction of T-lymphomas by x-rays is not understood. Haas has identified an early phase in which the T-lymphoma cells are growth factor-dependent and produce their own growth factor (autocrine phase) and a later phase, when the cells progress from growth factor-dependence to growth factor-independence. He grew cell lines from primary x-ray-induced thymic lymphomas (PXTLs) under conditions which minimize the progression of the cells from factor-dependence to factor-independence. All 22 PXTL lines grown secrete a growth factor which supports their own growth and which was named lymphoma growth factor (LGF). LGF-dependent cells are nontumorigenic or poorly tumorigenic, do not clone in soft agar, have no detectable rearrangements in the c-myc or Pim-1 region and possess near diploid or pseudodiploid karyotypes without evidence for trisomy of chromosomes 15 or 17. Using a variety of biochemical and functional assays, it appears that LGF is not related to any known growth factors. Its properties resemble those of a growth factor produced by virus-induced T-cell lymphomas of AKR mice and designated lymphoma-derived growth factor (LDGF) by Hays et al. (1984). Upon progression, PXTL cells become growth factor-independent, are highly tumorigenic *in vivo*, clone in soft agar, and assume a near triploid karyotype containing numerous chromosomal aberrations. Thus, these studies of Haas show that in x-ray-induced lymphomagenesis an autocrine, LGF-dependent phase precedes the progressive phase characterized by rearrangements in the myc and/or Pim-1 regions as well as by many chromosomal aberrations (47).

Weinberg has previously shown that neither a ras nor a myc oncogene can transform rat embryo fibroblasts when acting alone; however, the two genes acting in concert are able to do so. This suggests that the two genes act in distinct and complementary ways on the cellular phenotype and makes possible a procedure by which yet other oncogenes can be tested for their ability to collaborate with either ras or myc in transformation. Following this procedure, he has found that appropriately constructed clones of the src oncogene can collaborate with myc in transformation, while clones of the p53, myb, and ski oncogenes can collaborate with ras in transformation. This extends the catalog of oncogenes that collaborate with ras to transform cells to include c-myc, N-myc, adenovirus E1A, polyomavirus large T-antigen, SV40 large T-antigen, p53, myb, and ski. All these oncogenes appear to function analogously. Significantly, the gene products of all these genes are found in the nucleus. Conversely, the oncogenes collaborating with myc include ras, polyoma middle T-antigen and src. The proteins of these oncogenes are all found in the cytoplasm. These observations suggest that there are two central regulatory pathways governing cell growth which must be affected by oncogene proteins in order for transformation to ensue (105,106).

In other studies, Weinberg examined the effects of the myc oncogene on the cell's responsiveness to exogenous mitogenic growth factors. He found in a collaborative study with Drs. A. Roberts and M. Sporn of the NCI that the myc oncogene sensitizes Rat-1 cells to the growth factor EGF. Whereas the Rat-1 cells are unable to grow in soft agar either in their normal genotype or after acquisition of a myc oncogene, myc-bearing cells acquire the anchorage independence phenotype in the presence of EGF. This response is specific for EGF and is not exhibited by PDGF or a number of other growth factors. This demonstrates that one effect of myc is to alter the cell's response to a growth factor.

Moreover, it may provide a mechanistic explanation for the observed cooperation between ras and myc oncogenes. Ras-like oncogenes induce the cell to secrete EGF-like factors (e.g., TGF-alpha) while myc causes the cell to be more responsive to the recently secreted factors, thereby creating a strongly acting positive feedback (autocrine) loop of growth control (105,106).

A similarity exists between work of Weinberg which demonstrates a requirement for two genetically activated oncogenes in tumorigenesis and the work of others that shows that during initiation-promotion carcinogenesis, two genetic alternations must occur in order for tumor growth to ensue. The first lesion creates the "initiated" cell while the second lesion converts the promoter-dependent papillomatous cell into an autonomously growing carcinoma. Weinberg has suggested that the creation of an initiated cell requires the activation of a ras or a myc oncogene and that such an initiated cell, once created, should be especially responsive to the growth promoting effects of a tumor promoter like TPA (tetradecanoylphorbol acetate). He made the additional assumption that rat embryo fibroblasts can serve as good models for the much-studied skin keratinocytes of the initiation-promotion experimental system. He has found that a ras, but not a myc oncogene can elicit a cell phenotype that mimics at least some of the aspects of initiated state observed *in vivo*. A rat embryo fibroblast carrying a ras oncogene is sensitized to the mitogenic effects of several tumor promoters which promote the clonal outgrowth of the oncogene-bearing cell. Retinoic acid, which blocks promotion *in vivo*, is also effective in blocking clonal expansion *in vitro*. He is further developing this system in an attempt to determine whether all aspects of initiation-promotion can be studied in this *in vitro* system (105,106).

Two RFAs for traditional research grants were initiated by the RNA I component in FY 85 and were funded this year. The first research grant initiative concerned basic studies on the development and assessment of retroviral vaccines. It was based on a BCB workshop held at NIH on December 10-11, 1984. The RFA was designed to stimulate both *in vitro* and *in vivo* experimental studies to develop and/or assess new retroviral vaccines. It is anticipated that four new research projects will be funded as a result of the responses received to this RFA. The second research grant initiative concerned studies of novel exogenous and endogenous human retroviruses and was based on a discussion group which met on March 11, 1985 at NIH in Bethesda. The RFA was designed to stimulate research to isolate and characterize these novel human retroviral entities and determine their significance in human cancer. It is anticipated that four new research projects will be funded as a result of the responses received to this RFA.

In addition, a program announcement concerning feline leukemia virus was issued in FY 85. It was based on input from a BCB workshop held on November 29-30, 1984 at the NIH. This program announcement was designed to stimulate additional studies on the biology, immunology, and molecular biology of feline leukemia virus. This announcement stimulated the submission of a single R01 application. That application received a sufficient priority score to be funded from the grant pool this year.

On March 28, 1986, the DNA I and RNA I components of the Biological Carcinogenesis Branch co-sponsored a discussion group on "Vector-Mediated Regulation of Gene Expression." The purpose of this discussion group was to ascertain the state of

the art in the use of anti-sense RNA, in particular, for gene regulation and its possible relevance to the regulation of genes expressed in virus-transformed cells. The consensus of the group was that there had not been a systematic approach to study the mechanism of action of anti-sense RNA or to investigate the construction of vectors used to express the anti-sense sequences in cells. It is planned to have the results of this discussion group presented at the October 1986 meeting of the DCE Board of Scientific Counselors and to propose an RFA targeted to these specific issues.

Thus, the investigations administered by the RNA I component have focused on studies to elucidate the biology and diverse characteristics of mammalian retroviruses and on their interactions with and/or perpetuation in their host cells resulting in the transformation of normal cells into the malignant phenotype. Evidence was obtained that carcinogenesis by human T-cell lymphotropic virus I and II involved a trans-activation mechanism under the direct control of the viral gene termed the tat gene. A similar mode of action was proposed for viral replication and induction of the AIDS syndrome by the HTLV-III/LAV family of viruses. New viruses of this latter category, termed STLV-III, were demonstrated to play an etiological role in an AIDS-like syndrome in rhesus monkeys. Apparently normal African green monkeys and humans in Senegal, West Africa, were found to be chronically infected with a latent nonpathogenic variant of this simian AIDS virus STLV-III. A feline retrovirus isolate, with reproducible capacity to induce a rapid and fatal AIDS syndrome in cats, was isolated and characterized. Recombinant DNA techniques were exploited for defining the cellular and/or viral sequences involved in conferring tissue tropism to virus, type of cancer induced and in conferring the ability of viruses to replicate and induce cell transformation. Oncogenesis by specific products of oncogenes was studied using deletion mutants and recombinants. The involvement of and collaboration between two or more oncogenes in carcinogenesis was explored and defined.

RNA VIRUS STUDIES I
GRANTS ACTIVE DURING FY86

<u>Investigator/Institution/Grant Number</u>	<u>Title</u>
1. ARLINGHAUS, Ralph B. Scripps Clinic and Research Foundation 2 R01 CA 36714-02A2	Moloney Sarcoma v- <u>mos</u> Proteins in Transformation
2. AXEL, Richard Columbia University (New York) 5 P01 CA 23767-08	Molecular Virology
3. BACHELER, Lee T. Temple University 2 R01 CA 29519-05A1	Organization and Expression of Leukemia Virus Genomes
4. BALTIMORE, David Whitehead Institute for Biomedical Research 5 P01 CA 38497-02	Interactions of Oncogenes with Developing Systems
5. BARON, Samuel University of Texas Medical Branch (Galveston) 5 U01 CA 40764-02	Host Defenses Against HTLV I and II
6. BEDIGIAN, Hendrick G. Jackson Laboratory 5 R01 CA 31102-06	A New Murine Model for the Study of Myeloid Leukemia
7. BESMER, Peter Sloan-Kettering Institute for Cancer Research 5 R01 CA 32926-03	Oncogenes of New Sarcoma Virus Strains
8. BOETTIGER, David E. University of Pennsylvania 1 R01 CA 39473-01	Models for Leukemogenesis by <u>src</u> Containing Viruses
9. BRONSON, David L. Southwest Foundation for Biomedical Research 1 R01 CA 43361-01	Studies on a Novel Human Retrovirus
10. BROWN, J. Martin Stanford University 5 R01 CA 03352-30	Biological Aspects of Carcinogenesis by Radiation

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| 11. | CHEN, Irvin S. Y.
University of California
(Los Angeles)
5 R01 CA 38597-02 | A Molecular Genetic Study of
Human T-Cell Leukemia Virus |
| 12. | COHEN, James C.
Louisiana State University
Medical Center (New Orleans)
5 R01 CA 34823-05 | The Molecular Genetics of
Retroviruses |
| 13. | COHEN, James C.
Louisiana State University
Medical Center (New Orleans)
5 R01 CA 35686-03 | Retrovirus Sequence Specific
Integration in Human Cells |
| 14. | COMPANS, Richard W.
University of Alabama
(Birmingham)
2 R01 CA 18611-12 | Directional Transport of MuLV
Glycoproteins |
| 15. | COOPER, Geoffrey M.
Dana-Farber Cancer Institute
5 R01 CA 18689-11 | Infectious DNA for Endogenous
RNA Tumor Virus Genes |
| 16. | DARNELL, James E., Jr.
Rockefeller University
5 P01 CA 18213-11 | Correlated Program in Viral
Oncology |
| 17. | DE FRANCO, Donald B.
University of Pittsburgh
1 R01 CA 43037-01 | Glucocorticoid Regulation of
Retroviral Transcription |
| 18. | DESROSIERS, Ronald C.
Harvard Medical School
5 U01 CA 40680-02 | T-Cell Lymphotropic Viruses of
Macaques |
| 19. | DONEHOWER, Lawrence A.
Baylor College of Medicine
1 R01 CA 41476-01 | Role of Virus and Cell Genes in
Retrovirus Replication |
| 20. | DONOGHUE, Daniel J.
University of California
(San Diego)
5 R01 CA 34456-03 | Expression of Retroviral
Envelope Gene Fusion Proteins |
| 21. | DUDLEY, Jacquelin P.
University of Texas (Austin)
5 R01 CA 34780-03 | Amplification of MMTV DNA in
T-Cell Lymphomas |

33. FAN, Hung Y.
University of California (Irvine)
5 R01 CA 32455-06
Expression of C-Type Virus Genes
34. FERRER, Jorge F.
University of Pennsylvania
5 R01 CA 40688-02
Regulation of BLV Infection and Leukemogenesis
35. FIRESTONE, Gary L.
University of California
(Berkeley)
5 R01 CA 35547-03
Steroid Regulation of Protein Maturation
36. FLEISSNER, Erwin J.
Sloan-Kettering Institute for
Cancer Research
5 P01 CA 16599-12
Hematopoietic Cell Transformation by Retroviruses
37. FLYER, David C.
Pennsylvania State University
Hershey Medical Center
7 R23 CA 44633-01
Specificity of the CTL Response to Murine Leukemia Virus
38. FRIEND, Charlotte
Mount Sinai School of Medicine
5 R01 CA 10000-20
Filterable Agents and Tumor Induction in Mice
39. GARDNER, Murray B.
University of California (Davis)
5 R01 CA 30912-03
Mammary Tumorigenesis in Hosts Lacking MuMTV DNA
40. GARVER, Frederick A.
Medical College of Georgia
3 R01 CA 32578-04S1
Characterization of Tumor Antigen on Leukemia Cells
41. GATTONI-CELLI, Sebastiano
Massachusetts General Hospital
1 R01 CA 43499-01
Human Endogenous Retroviruses in Colon Cancer
42. GOFF, Stephen P.
Columbia University (New York)
5 R01 CA 30488-06
Construction and Analysis of Retrovirus Mutants
43. GOLDSCHNEIDER, Irving
University of Connecticut Health
Center
5 R01 CA 38762-02
Cellular Targets of Leukemic Transformation
44. GRANT, Christopher K.
Pacific Northwest Research
Foundation
1 R01 CA 43371-01
Anti-Idiotypic Vaccines for Feline Leukemia Virus

45. GREEN, William R.
Dartmouth College
1 R01 CA 43475-01
Study on Development and Assessment of Retroviral Vaccines
46. GUPTA, Phalguni
University of Pittsburgh
5 U01 CA 42732-02
Mechanism of Action of a Nonantibody BLV Blocking Protein
47. HAAS, Martin
University of California
(San Diego)
5 R01 CA 34151-05
Viral Malignant Lymphomagenesis in X-Irradiated Mice
48. HAAS, Martin
University of California
(San Diego)
1 R01 CA 42432-01
Studies of an Africa Population Endemic for HTLV
49. HARFORD, Esther C.
Uniformed Services University
of the Health Sciences
2 R01 CA 34582-04
Oncogenes in Human Cancer Induction
50. HASELTINE, William A.
Dana-Farber Cancer Institute
5 R01 CA 19341-10
The Molecular Biology of Replication RNA Tumor Viruses
51. HASELTINE, William A.
Dana-Farber Cancer Institute
5 R01 CA 36974-03
Study of pX Region of Human T-Cell Leukemia Virus
52. HAYS, Esther F.
University of California
(Los Angeles)
5 R01 CA 12386-13
Development of Lymphoma in the Thymus
53. HOLLAND, Christie A.
University of Massachusetts
Medical School
1 R01 CA 41510-01
Determinants of the Oncogenic Potential of MCF Viruses
54. HOOVER, Edward A.
Colorado State University
5 R01 CA 32552-02
Pathogenesis of Animal Leukemia
55. HOPKINS, Nancy H.
Massachusetts Institute of
Technology
5 R01 CA 19308-11
Studies on Endogenous and Other C-Type Viruses of Mice

56. HUNTER, Anthony R.
Salk Institute for Biological
Studies
5 R01 CA 17096-11
Macromolecular Synthesis and
Growth Control
57. HUNTER, Anthony R.
Salk Institute for Biological
Studies
5 R35 CA 39780-02
Role of Protein Phosphorylation
in Growth Control
58. HUNTER, Eric
University of Alabama
(Birmingham)
2 R01 CA 27834-06
Genetics of Primate "D" Type
Retroviruses
59. KABAT, David
Oregon Health Sciences University
5 R01 CA 25810-08
Leukemogenic Membrane
Glycoproteins: gp55s of SFFVs
60. LERNER, Richard A.
Scripps Clinic and Research
Foundation
5 P01 CA 27489-07
Consequences of Endogenous
Retroviral Expression
61. LEVY, Jay A.
University of California
(San Francisco)
5 R01 CA 33137-03
Role of Endogenous Xenotropic
Viruses
62. LILLY, Frank
Yeshiva University
5 R01 CA 19931-09
Mechanism of the H-2 Effect on
Viral Leukemogenesis
63. LUFTIG, Ronald B.
Louisiana State University
Medical Center (New Orleans)
5 R01 CA 37380-04
Assembly of Murine Leukemia
Viruses
64. MERUELO, Daniel
New York University
5 R01 CA 22247-09
Genetics of Resistance to
Leukemia
65. MERUELO, Daniel
New York University
2 R01 CA 31346-04A1
Study of MuLV Sequences in the
MHC: Cloning of Minor H Genes
66. MULLINS, James I.
Harvard University
5 U01 CA 40646-02
Viral Determinants of HTLV-I
Leukemogenesis

67. MUNDY, Gregory R.
University of Texas Health Science
Center (San Antonio)
5 U01 CA 40690-02
Human T-Cell Leukemia Lymphoma
Virus
68. MURPHY, Edwin C., Jr.
University of Texas System
Cancer Center
2 R01 CA 34734-04
MuSV Ts110: Thermosensitive
RNA Splicing in Intact Cells
69. NEIL, James C.
Beatson Institute for Cancer
Research, Glasgow, Scotland
1 R01 CA 40034-01
The Role of Feline Leukemia
Viruses in Anemia
70. NEURATH, A. Robert
New York Blood Center
1 R01 CA 43315-01
Synthetic HTLV-III env Protein
Analogues for Future Vaccines
71. O'DONNELL, Paul V.
Sloan-Kettering Institute for
Cancer Research
5 R01 CA 31491-05
Kinetic Study of Virus-
Accelerated Leukemia
72. OLSEN, Richard G.
Ohio State University
5 R01 CA 30338-06
FeLV Leukemogenesis and Pre-
neoplastic Lesions
73. OLSEN, Richard G.
Ohio State University
5 U01 CA 40714-02
Immunoprevention of HTLV
Infection
74. PALKER, Thomas J.
Duke University
5 U01 CA 40660-02
Monoclonal Antibodies to HTLV-I
and HTLV-II
75. PETERSON, David O.
Texas A and M Research
Foundation
2 R01 CA 32695-04A1
Genetic and Molecular Analysis
of Steroid Responsiveness
76. PINTER, Abraham
Public Health Research Institute
of the City of New York
5 R01 CA 42129-02
Biochemical and Genetic Studies
of MuLV Envelope Proteins
77. RACEVSKIS, Janis
Sloan-Kettering Institute for
Cancer Research
1 R01 CA 39509-01
MMTV Gene Products and
Transformation

78. RACEVSKIS, Janis
Montefiore Medical Center
(New York)
7 R01 CA 43864-01
MMTV Gene Products and Transformation
79. RADKE, Kathryn
University of California (Davis)
5 U01 CA 40653-02
Cellular Transformation by Bovine Leukemia Virus
80. RASHEED, Suraiya
University of Southern California
5 R01 CA 27246-06
Leukemia and Sarcoma Genes in Cellular Transformation
81. RISSER, Rex G.
University of Wisconsin (Madison)
1 R01 CA 41302-01
Biological and Molecular Studies of A-MuLV Tumorigenesis
82. ROJKO, Jennifer L.
Ohio State University
5 R01 CA 35747-03
Pathobiology of Latent Lymphomagenic Feline Retroviruses
83. ROSENBERG, Naomi E.
Tufts University
5 R01 CA 24220-08
Abelson Leukemia Virus Transformation
84. ROSENBERG, Naomi E.
Tufts University
5 R01 CA 33771-04
RNA-Tumor Virus--Hematopoietic Cell Interaction
85. ROY-BURMAN, Pradip
University of Southern California
5 R01 CA 40590-02
Endogenous Retrovirus Related Genes in Feline Leukemia
86. SARKAR, Nurul H.
Sloan-Kettering Institute for Cancer Research
5 R01 CA 17129-12
Components of the Murine Mammary Tumor Virus
87. SCHWARTZ, Robert S.
Tufts University
5 P01 CA 24530-07
Experimental Leukemogenesis
88. SEFTON, Bartholomew M.
Salk Institute for Biological Studies
1 R01 CA 42350-01
Thymoma Tyrosine Protein Kinase
89. SHERR, Charles J.
St. Jude Children's Research Hospital
5 R01 CA 38187-03
The fms Oncogene

90. SODROSKI, Joseph G.
Dana-Farber Cancer Institute
5 U01 CA 40658-02
Role of HTLV LOR Region in
Transcriptional Regulation
91. SODROSKI, Joseph G.
Dana-Farber Cancer Institute
5 U01 CA 40659-02
Expression and Potential
Function of BLV LTR Region
92. SORGE, Joseph A.
Scripps Clinic and Research
Foundation
5 R01 CA 36448-03
Gene Transfer and Expression
Using Retroviruses
93. SRINIVAS, Ranga V.
University of Alabama
(Birmingham)
5 R01 CA 40440-02
Site-Specific Modification of
SFFV Glycoproteins
94. STEFFEN, David L.
Worcester Foundation for
Experimental Biology
5 R01 CA 30674-05
Mechanisms of Viral and
Nonviral Leukemogenesis
95. TAKETO, Makoto
Jackson Laboratory
1 R01 CA 39652-01
Viral Gene Expression in
Embryonal Carcinoma Cells
96. THOMAS, Christopher Y.
University of Virginia
(Charlottesville)
2 R01 CA 32995-04A1
Molecular Genetics of Leukemia
Viruses of Inbred Mice
97. VAIDYA, Akhil B.
Hahnemann University
5 R01 CA 22413-09
Etiological Studies of Mammary
Carcinoma
98. VAN BEVEREN, Charles P.
La Jolla Cancer Research Foundation
1 R01 CA 42909-01
Proto-Oncogene *fms*: Activation
and Normal Function
99. VARMUS, Harold E.
University of California
(San Francisco)
5 R01 CA 19287-10
Molecular Biology of Mouse
Mammary Tumor Virus
100. VERMA, Inder M.
Salk Institute for Biological
Studies
5 R01 CA 16561-12
Viral and Cellular Oncogenes

101. VERMA, Inder M.
Salk Institute for Biological
Studies
2 R01 CA 21408-10
Retroviral Vectors: Gene
Transfer
102. VOGT, Marguerite M.
Salk Institute for Biological
Studies
2 R01 CA 13608-14
Viral Gene Functions Involved
in Transformation
103. VOLSKY, David J.
University of Nebraska (Omaha)
1 R01 CA 43464-01
Novel Retrovirus from South
America: HTLV-Type Viruses
104. WACHSMAN, William
University of California
(Los Angeles)
1 R01 CA 43370-01
Human Retroviruses and Hairy-
Cell Leukemia
105. WEINBERG, Robert A.
Whitehead Institute for
Biomedical Research
5 R35 CA 39826-02
Molecular Basis of
Carcinogenesis
106. WEINBERG, Robert A.
Whitehead Institute for
Biomedical Research
5 R01 CA 39963-02
Construction of Novel Sarcoma
Virus Genomes
107. WEISSMAN, Bernard E.
Children's Hospital of Los
Angeles
5 R01 CA 36936-02
Retroviral Interaction with
Epidermal Keratinocytes
108. WEISSMAN, Irving L.
Stanford University
5 R01 CA 32031-05
The Receptor-Mediated Leuke-
mogenesis Hypothesis
109. WILSON, Michael C.
Scripps Clinic and Research
Foundation
2 R01 CA 33730-04
Regulation of Endogenous
Retroviral Gene Expression
110. WITTE, Owen N.
University of California
(Los Angeles)
2 R01 CA 27507-07
Transformation by Abelson
Murine Leukemia Virus

111. WONG, Paul K.
University of Illinois
(Urbana-Champaign)
5 R01 CA 36293-03
Molecular Basis of Pathogenesis
Induced by MLV Mutants
112. YOSHIMURA, Fayth K.
Fred Hutchinson Cancer Research
Center
5 R01 CA 25461-06
DNA Forms of Murine Leukemia
Viruses
113. YOSHIMURA, Fayth K.
University of Washington
7 R01 CA 44166-01
DNA Forms of Murine Leukemia
Viruses

SUMMARY REPORT

RNA VIRUS STUDIES II

The RNA Virus Studies II component of the Branch primarily involves studies of the avian tumor viruses and hepatitis B virus. This program consists of 99 research grants with an estimated total funding of 17.1 million dollars for FY86. Of these, approximately 84% are involved with studies of avian tumor viruses, and 12% concern hepatitis B virus or other hepatitis viruses and their relationship to primary hepatocellular carcinoma. The remaining 4% deal with a variety of agents which are less closely related to human diseases. The majority of studies funded by RNA Virus Studies II are focused on the molecular nature of the transformation process, the definition and discovery of new oncogenes (genes responsible for the transformation of cells from normal to malignant), and development and testing of hypotheses about the mechanism(s) of oncogenesis of viruses lacking oncogenes. In addition to 86 traditional R01 grants and four P01 program project grants, this component now includes five R35 Outstanding Investigator Awards as well as one R13 Conference Grant and three R23 New Investigator Awards.

One important question which investigators in avian retrovirology are attempting to answer is an elucidation of the mechanism(s) by which the protein products of viral oncogenes (v-onc), as well as those of their cellular counterparts (c-onc), initiate and maintain the transformed state. In general, much less is known about the functions of oncogene products than about the structure of oncogenes, and yet, knowledge of these functions in the normal cell and their possible modification in the malignant cell is essential to an understanding of the transformed cell phenotype. As the techniques for identifying and isolating oncogene products develop further, progress in the search for the essential transforming function of oncogene products will also be made.

A broad functional classification of oncogene products can be made by considering their location in the cell: at the cell surface, in the cytoplasm, and in the nucleus. An example of a cell surface onc protein is the product of the erb-B gene which is important in avian erythroblastic leukemia. A striking homology between the erb-B gene product and the EGF (epidermal growth factor) receptor has recently been found. The abl and mil oncogene proteins are localized in the cytoplasm of the transformed cell. The abl protein has a tyrosine specific protein kinase activity that, based on the behavior of viral mutants with defects in the abl gene, is important in leukemic transformation. The mil protein binds to DNA or RNA in vitro, thus possibly indicating a regulatory function for this protein. Interestingly, it is also related by nucleic acid sequence to the kinase family although, like the erb-B protein, it lacks kinase activity. The myc protein is localized in the nucleus and binds to double-stranded DNA in vitro. It is not known whether this binding is sequence specific. Expression of this protein results in a transcriptional activation of a large block of cellular genes. More recent work suggests that the myc protein can at least partially substitute for the function of the E1A gene product of adenovirus and can activate late gene expression of adenovirus as well as expression of a cellular heat shock protein. However, there are also data which indicate a negative regulatory effect of myc, and in which in vitro transcription was shown to be turned off by myc protein. Whether this latter effect is a result of binding to DNA is not yet known. Oncogenes show a certain, although imperfect, tissue tropism. For example, the erb-B oncogene-containing viruses

induce erythroblastic leukemia in the chicken. Myb-containing viruses cause myeloblastosis. The spectrum of tumors associated with the viral myc oncogene is much broader, including tumors in myelocytic elements as well as several different types of carcinomas. The abl oncogene transforms various types of lymphocytes. The basis for oncogene tissue tropism is not understood, although some investigators think that oncogene products are stage-specific differentiation proteins that prevent further progression of a cell along a predetermined differentiation pathway. Selected examples of the types of studies accomplished within the last year follow.

Recent studies from Bishop's laboratory have characterized a temperature-sensitive mutant of avian myeloblastosis virus and found that the mutation selectively effects the genesis of the sub-genomic mRNA (messenger RNA) from which the product of the viral oncogene v-myb arises. As a consequence of this defect, the production of the viral transforming protein was reduced below the threshold required for leukemogenesis. These findings provide the first direct genetic evidence to implicate v-myb in transformation; in addition, they have demonstrated a potentially novel defect in either the stability or biogenesis of viral messenger RNA--perhaps a conditional lesion in splicing.

Having obtained a complete nucleotide sequence of c-fps, this group was able to suggest that the transduction of this gene into the PRC-II strain of avian sarcoma virus was mediated in part by recombination within the poly(A) track at the 3' end of the mRNA for c-fps. They have obtained direct evidence for this suggestion and thus, the first evidence that right hand recombination during the course of transduction by retroviruses utilizes RNA rather than DNA molecules.

Two other systems which promise to give much additional useful data have been developed by this group. In the first of these, they have undertaken a search for tyrosine kinase genes in yeast. As a result of these studies, the group has found three closely related genes whose nucleotide sequences suggest that they encode a new type of protein kinase not previously described in yeast. Neither the products of these kinase genes nor their substrate specificities have been determined; they do not appear to be counterparts of any known proto-oncogene, although they are generally related to all of the tyrosine protein kinases by sequence homology. The finding of this new family of protein kinase genes in yeast will allow these investigators to use the versatile and detailed genetic analysis of yeast to better define the kinds of interactions that occur between protein kinases and their substrates in eukaryotic cells.

The second novel system involves the genetic and molecular analysis of the fruit fly, *Drosophila melanogaster*, as a principle strategy for determining the physiological functions of proto-oncogenes. Analysis of organisms and cells with genetic lesions affecting c-src, the highly conserved progenitor of the v-src gene of Rous sarcoma virus can provide insight into the physiological functions of c-src. Since such genetic analysis is presently impossible to perform with vertebrate organisms, these investigations were undertaken using the fruit fly. The complete nucleotide sequence of the *Drosophila* counterpart of src has been obtained and reveals extensive similarity between the *Drosophila* and human src products. It appears that the *Drosophila* gene encodes a 62 kD protein, which is remarkably similar to the protein product of chicken c-src. The *Drosophila* c-src locus is transcribed into three mRNAs that are each regulated independently during development. The *Drosophila* c-src mRNA is abundant in embryos and pupae, but rare in larvae and adults. Its expression during

embryologic development occurs first throughout the organism, but then becomes localized to muscular and neural tissue. In situ hybridization reveals that after the first eight hours of development, c-src mRNA accumulates almost exclusively in neural tissues such as the brain, ventral nerve cord, and eye antenna disks and in differentiating smooth muscle tissue. The temporal and tissue-specific regulation of c-src suggests that the gene is not solely a mitotic signal, but may have another function in differentiated cells that are no longer dividing; it may also play a role in the development of neural tissue and smooth muscle. Similar studies are now in progress using the *Drosophila* counterparts of c-erb-B (the EGF receptor), c-fps, and another kinase gene which has been isolated from *Drosophila*, but which at present has no apparent counterpart among known proto-oncogenes. The *Drosophila* counterpart of c-myb has been identified, isolated and sequenced. Study of this gene holds great interest, because it is presently the only representative of the proto-oncogenes encoding nuclear proteins that has been found in *Drosophila*.

Studies on the regulation, products and function of myc genes have revealed abnormalities in the regulation of c-myc which have been implicated in the genesis of various human malignancies. Two genetic domains located upstream of human c-myc have been identified: one apparently serves as an activator region of the gene, the other as a repressor. Additional studies are underway to determine how these two domains interact to control the action of c-myc and to try to identify cellular factors that might act on these domains.

The protein encoded by human c-myc has been isolated and characterized; it has been found to have an exceptionally brief half-life, to be located in the nucleus, and to bind to DNA. These are all properties consistent with speculation that the protein somehow regulates the expression of a battery of cellular genes. However, previous suggestions that a shut-off of c-myc is the precipitating event or is essential for the differentiation of mouse erythroleukemia cells have been refuted in these studies. Previous suggestions that myc and the adenovirus gene E1A are physiologically related has been strengthened by the construction of hybrids between the two genes: the results indicate that the two genes contain domains that complement one another to give full biological function. The biological capabilities of c-myc have been further assessed by incorporating the gene into a murine leukemia virus retroviral vector. In this form, the gene can transform established lines of rodent cells to a tumorigenic phenotype, sustaining the view that "deregulation" of c-myc can contribute to natural tumorigenesis.

Further studies on the function of other oncogenes have revealed that the products of both v-myb and c-myb have exceptionally brief half-lives, are located in the nucleus and bind to DNA. These newly described properties increase the likelihood that myc and myb genes serve related physiological functions, although in different tissues. The complete nucleotide sequence of chicken c-myb has been obtained, and the gene has been inserted into a murine leukemia virus retroviral vector so that the biological activity of the gene could be tested in cultured cells and in rodents.

The isolation of c-DNAs representing the complete c-erb-B genes (EGF receptor) has been reported. These will be used to make hybrid genes with the gene encoding the insulin receptor and with the gene encoding the receptor for the hematopoietic growth factor CSF1 (c-fms). These hybrids will be used in an attempt to localize the specificity of action within the various receptor

proteins and to dissect elements of their biogenesis. The protein encoded by v-erb-B has been found to have intrinsic tyrosine protein kinase activity; the substrate specificity of this kinase differs from that of the src protein, another oncogene product with tyrosine protein kinase activity. The v-erb-B gene has been inserted into a murine leukemia virus retroviral vector, in which form it transforms murine fibroblasts and erythroid cells that produce presently uncharacterized hematopoietic tumors in rodents.

The kind of lymphoid cell transformed by the v-rel oncogene has only recently been defined. Molecular clones have been prepared for the heavy and lambda-light chains of chicken immunoglobulins (Ig) and used to characterize bone-marrow cells transformed by v-rel. Phenotypically, none of the transformed cells express Ig genes; however, the cells display three distinct Ig genotypes: germline configuration; one heavy chain and one light chain allele rearranged to what appears to be the same abnormal configuration found in numerous different clones of transformed cells; and homo/hemi-zygosity for the abnormal rearrangements. These findings raise the possibility that the cells transformed by v-rel represent previously unidentified early progenitors in the B cell lineage or novel abnormal rearrangements of Ig genes.

The nucleotide sequence of the human locus of the newly described N-myc proto-oncogene has been obtained. The gene is closely related to c-myc and is expressed in a controlled manner during the course of mouse embryogenesis and in a restricted manner in adults, particularly in neural tissues. Amplification and over-expression of N-myc are common abnormalities in the advanced stages of human neuroblastoma; these measurements of N-myc appear likely to be useful in the diagnosis and management of this disease. The product of N-myc has been identified; it is a nuclear protein that has a short half-life and binds to DNA, properties consistent with membership in the burgeoning myc gene family. Additionally, it has been shown that N-myc can transform cultured cells in two settings: rat embryo cells in cooperation with mutant c-Ha-ras, and established rodent fibroblasts without assistance. Both forms of transformation require vigorous expression of N-myc, but no intrinsic abnormality of the gene. These findings establish N-myc as an authentic proto-oncogene and strengthen the suspicion that its amplification can contribute to the progression of human neuroblastoma. Molecular constructs containing "anti-sense" RNA have been made which allow stable repression of gene expression in cultured cells. This work was first accomplished in pilot studies in which herpesvirus thymidine kinase was the repressed gene and has now been extended to work with N-myc in the hope of obtaining direct evidence to implicate this gene in tumorigenesis.

Finally, using a gene transfer assay, it has been possible to demonstrate consistent activation of a c-ras gene in clinical samples of human chronic myelogenous leukemia (CML) from both chronic and blast crises phases of the disease. If confirmed by further work, this finding would represent the first time that abnormalities of a ras gene have been found consistently in one type of human malignancy (5).

Additionally, studies by Hanafusa, et al., have investigated the cellular fps gene. The viral counterpart of this gene was first found in the Fujinami sarcoma virus, a replication-defective avian sarcoma virus, whose genome contains a unique sequence of approximately 2,700 nucleotides called v-fps. The sequence encodes a transforming protein of 1,182 amino acids, P130, which exhibits tyrosine kinase activity and is phosphorylated in vivo. Its cellular

counterpart, c-fps, has been detected in the DNA of uninfected cells of various vertebrate species. In the avian system, the sequence was shown to be expressed in a tissue-dependent manner. The c-fps gene product was identified as a phosphoprotein of 98 kD molecular weight and designated as NCP98. This protein has tyrosine kinase activity and is structurally related to the viral transforming protein P130. Expression of NCP98 kinase activity is tissue specific, with a predominance of activity in bone marrow. Moreover, the NCP98 kinase activity is high in avian myeloblastosis virus-transformed myeloid cells, but low in avian erythroblastosis virus-transformed erythroid cells. These observations suggested that c-fps might be differentially expressed in some hematopoietic cells. To identify hematopoietic cells which preferentially expressed NCP98, the expression of this protein was analyzed in purified hematopoietic cell populations and in hematopoietic tissues during the development of chickens.

It was demonstrated that in bone marrow, spleen, and bursa, maximum NCP98 kinase activity on a per cell basis correlated with the peak of granulopoiesis in these organs. Furthermore, in a bovine serum albumin density gradient fractionation of bone marrow cells, granulocytic cells appeared to account for most of the NCP98 kinase activity. No correlation was found between the distribution of erythrocytic, lymphocytic or thrombocytic cells and the distribution of expression of NCP98 kinase activity. However, NCP98 protein and kinase activity were ten-fold higher in macrophages than in bone marrow. In addition, depletion by complement mediated lysis of erythrocytic cells in bone marrow did not significantly reduce the total recovery of NCP98 kinase activity. These results argue for the specific expression of a c-fps gene product in granulocytic cells and macrophages.

It is possible to imagine that this protein is involved in some functions specific to these cells. Both cell types are phagocytic. In mice and humans, granulocytes and macrophages are closely related and are derived from a common progenitor cell. Although there is no evidence for a similar relationship between the two lineages in chickens, it is possible that NCP98 plays some role in the differentiation of these cells. Alternatively, NCP98 may have some relation to growth factors or the receptors specifically required for these hematopoietic cells. Fujinami sarcoma virus has been described essentially as an agent inducing sarcomas *in vivo* and transforming fibroblasts *in vitro*. In light of the results of this work, the effects of the v-fps protein on macrophages and granulocytic cells *in vivo* and *in vitro* should be reinvestigated. Such studies could yield pertinent information on the function of NCP98 during the differentiation of these cells.

Many transforming retroviruses carry transforming genes derived from transduced cellular genes fused with the viral gag gene. However, no cellular gene expressed as a fusion protein by a transforming retrovirus has yet been characterized for transforming potential without the attached gag gene sequences. It was previously demonstrated that gag gene sequences could be removed from the gag-fps fusion gene in Fujinami sarcoma virus, without reducing the transformation potential of this gene. However, removal of the gag-gene sequences did alter the morphological phenotype of the transformed cells. In addition, there have been reports that mutations in the gag portion of both the gag-fps gene of Fujinami sarcoma virus and the gag-abl gene of Abelson murine leukemia virus can alter some transformation parameters of these genes. These reports suggest that the gag gene sequences are not simply a vehicle for

expression of a cellular gene in a retrovirus. The Fujinami sarcoma virus-derived v-fps sequences that lack gag gene sequences had 25 amino acid differences with respect to the cellular gene. Thus, it was not clear whether c-fps sequences by themselves possessed transformation potential or whether the acquired mutation activated a transforming potential. Therefore, it was of interest to determine whether the gag gene sequences which were not specifically required for transformation could still be providing a mutagenic effect capable of activating a transformation potential of the c-fps gene. All data accumulated thus far suggests that cellular proto-oncogenes must be activated, either by elevated gene expression or by alteration of the primary structure of the gene product, or both, in order to transform cells.

In order to test these alternatives, c-fps gene sequences were substituted for v-fps gene sequences in two viral constructs: one that overexpressed a c-fps gene product that was indistinguishable from normal c-fps gene products expressed in bone-marrow cells and another that expressed c-fps sequences linked to gag gene sequences. These molecular constructs were used to determine the factors that are important for activating the transforming potential of the c-fps gene.

The constructs which contained viral gag sequences fused to either cellular or viral fps gene sequences were transfected into chick embryo fibroblasts with the appropriate helper virus DNA. Foci of transformed cells were detected after seven days. While the morphology of each of the transformed cells induced by the different constructs was distinct, definite transformation was observed with each type of construct. When virus recovered from each of the transformed cell lines was injected into the wing webs of seven-day-old chicks, tumors of comparable size were detected at the site of inoculation within seven days. When constructs without the gag sequences were treated similarly, neither was able to transform chicken cell fibroblasts as measured by focus formation and growth in soft agar. Transformation was not detected even after subculture of transfected cells for up to two months.

Control experiments carried out to determine if the absence of transformation was due to some trivial or confounding cause indicated that the amounts of the fps protein found in the chick embryo fibroblasts was approximately the same with the gag-fps and the fps constructs and that there were no major differences in stability between the different proteins. Thus, a lack of transformation in the transfected cells lacking the gag elements was not thought likely to be due to low levels of the protein in the affected cells or to the instability of the protein. However, the inability to induce transformation by transfection may have been the result of a cloning artifact. Therefore, the entire c-fps sequence used was excised and cloned into new constructs with the corresponding position in an entirely different clone containing viral gag sequences. The focus forming ability of these viral DNA clones was identical to that of the original constructs containing the c-fps sequences plus the viral gag sequences. They induced transformed cells within seven days. In addition, the cell extracts from these transformed cells possessed high levels of protein kinase activity. These results demonstrated that the c-fps sequences had not lost some intrinsic property required for transformation during the construct cloning. It was previously shown that v-fps gene sequences without gag sequences could transform chick embryo fibroblasts and induce tumors in chickens. It was shown here that the corresponding c-fps sequences lack the ability to transform chick embryo fibroblasts even when overexpressed by retroviral control elements.

There are 25 amino acid differences between the viral and cellular fps sequences expressed in these constructs. It is not known which amino acid changes contribute to the transforming activity of the v-fps sequences. However, several mutations may be required, since no transforming mutants of the gag negative c-fps constructs were obtained during two months of passage of infected cells. In contrast, spontaneous transforming mutants were recovered with high frequency from cultures infected with the c-src containing virus.

The level of protein kinase activity detected in extracts of c-fps transfected cultures was lower than that observed in the v-fps transfected cultures as measured by autophosphorylation in an in vitro protein kinase assay. This is similar to the observation with src, where the kinase activity of a cellular protein is reduced relative to the viral protein. Further analysis of the protein kinase activities of the fps proteins and in vivo substrates will be necessary in order to establish whether changes in kinase activity correlate with the activation of transforming activity. This study also demonstrated that the transformation potential of the c-fps gene could be activated when viral gag gene sequences were fused to its 5' end to produce a gag-fps fusion product. The gene product produced in these transfected cells is a gag-fps fusion protein with a protein kinase activity similar to that of Fujinami sarcoma virus. C-fps constructs transformed cells to unique morphologies reflecting differences between the viral and cellular fps coding sequences. The 5' addition of gag gene sequences to c-fps sequences includes 148 base pairs of v-fps-derived sequences that were removed in one of the vector constructions. However, this 148 base pair region most probably represents upstream, noncoding c-fps sequences; and therefore, it might contribute along with gag sequences to alter the phenotypic expression in the resulting virus.

There have been seven independent isolates of fps-containing transforming retroviruses with a gag-fps gene structure and none without a gag-fps gene structure. Thus, activation of the transformation potential of the c-fps gene by addition of gag gene sequences to the 5' end of a c-fps gene is relatively common. Although their overall structures are very similar to each other, the fps/fes-containing viruses that have been sequenced have different amounts of gag gene sequence attached to their amino termini and three or four have different amounts of cell derived sequences. The variability of amino terminal structures in fps/fes-containing retroviruses suggest that there are several amino terminal modifications that can activate the transforming potential of the c-fps gene. Most transforming retroviruses carrying transduced cellular genes encode gag fusion genes. The studies just described are the first demonstration that fusion with gag gene sequences actually provides a mutagenic event capable of activating the transforming potential of a cellular gene. This kind of mutagenic event may be analogous to the alteration generated by translocation of a cellular gene into the coding region of another cellular gene that is being expressed at its efficient levels or is subsequently amplified. Recently, it was shown that the c-abl gene is rearranged and amplified in a chronic myelogenous leukemic cell line K562. The c-abl protein, for which kinase activity has not yet been detected, acquired an amino terminal addition of 60 kd. This alteration apparently activated a tyrosine kinase activity in the c-abl gene product. It is conceivable that the transformation potential of the c-fps gene could be activated by the kind of translocation reported in K562 cells. Although such a translocation involving the c-fps gene has not been reported, there is evidence of higher than normal levels of expression of fps gene sequences in some human malignancies.

A final set of investigations from the Hanafusa group concerned the levels of phosphatidylinositol kinase in virally transformed and nontransformed cells. There has been a great deal of interest in the phosphatidylinositol kinase system because of the suggestions (Erickson, Balduzzi, Macara) of a direct involvement of oncogene products in this intracellular signaling system that utilizes inositol lipids and seems to be involved in regulating cell growth. These investigators reported that the purified transforming protein of Rous sarcoma virus (p60 v-src) has phosphatidylinositol (PI), phosphatidylinositol-4-monophosphate and diacylglycerol kinase activity. They further suggested that the increased phosphatidylinositol turnover is a direct effect of lipid kinase activity of p60 v-src and that the elevated PI turnover results in transformation through the overproduction of second messengers. It was shown that specifically immunoprecipitated UR2 transforming gene product (P68 gag-ros) was able to phosphorylate phosphatidylinositol. Thus, it was suggested that various oncogene products possessing tyrosine kinase activity might also have phosphatidylinositol kinase activity and that the PI kinase activity is responsible for transformation (25,2,52).

Therefore, these investigators wished to ask two cogent questions relating to the hypothesis that PI kinase activity might be responsible for transformation. They were: a) If elevated phosphatidylinositol turnover is a direct effect of the PI kinase activity of oncogene products, then what is the difference in the overall PI kinase activity between transformed and nontransformed cells, and in transformed cells how much of it is attributable to the PI kinase activity of the oncogene products? b) Is there any PI kinase activity associated with the oncogene products of the Fujinami sarcoma virus (FSV), P140 gag-fps which has tyrosine kinase activity?

To answer these questions, phosphatidylinositol kinase activity was assayed in detergent extracts of nontransformed or virally transformed cells. Nontransformed chick embryo fibroblasts contain phosphatidylinositol kinase activity with an apparent specific activity of 20 pmole/minute/mg protein which sedimented as a single peak with a molecular weight of approximately 60 kD in a glycerol gradient. Immunoprecipitation using anti-p60 src sera showed that the PI kinase activity was distinct from p60 c-src. Extracts from chick embryo fibroblasts transformed by Rous sarcoma virus, Fujinami sarcoma virus, or avian sarcoma virus UR2 showed no elevation of the PI kinase activity when compared with nontransformed chick embryo fibroblasts. Removal of the oncogene products from the extracts by immunoprecipitation did not change the level of the PI kinase activity in extracts, suggesting that putative virally coded PI kinases did not make a significant contribution to the overall levels of the PI kinase activity in transformed cells. These findings indicate that elevated phosphatidylinositol turnover in RSV and UR2 transformed cells is not the direct effect of PI kinase activity of oncogene products. Furthermore, the results indicate that in this cell system at least, viral transformation does not change the overall level of phosphatidylinositol kinase activity. This would indicate that activity(s) other than PI kinase activity is altered in transformed cells and is responsible for the elevation of phosphatidylinositol turnover in RSV or UR2 transformed cells (35).

Brugge has shown that a 90 kD protein, which is found complexing to the avian progesterone receptor in nontransformed cells, is indistinguishable from the 90 kD protein which associates in a complex with the Rous sarcoma virus transforming protein pp60 v-src. This identity was established by demonstrating that

monoclonal antibodies directed against the pp60 v-src-associated 90 kD protein recognized the 90 kD progesterone receptor binding protein in an immunoblast assay. Conversely, monoclonal antibodies that recognized a progesterone receptor binding protein were bound to the 90 kD protein which complexes with pp60. Secondly, peptide maps prepared from immunoprecipitates of either protein are indistinguishable. Thirdly, preincubation of the progesterone receptor complex with monoclonal antibodies prepared against pp60 v-src-associated proteins caused a shift in the sedimentation of the progesterone receptor. This 90 kD protein, which is indistinguishable from one of the major heat-shock proteins (which are induced under a variety of stress conditions in eukaryotic cells), may have an additional new role in the action of steroid hormones (10).

In studies carried out this year on the hepadnaviruses, a deletion in chromosome 11P associated with a hepatitis B virus (HBV) integration sites in hepatocellular carcinoma has been reported by Rogler. Hepatocellular carcinoma cells isolated from virus carriers often contain clonally propagated viral DNA integrations. As small chromosomal deletions are associated with several types of carcinomas, the occurrence of chromosomal deletions in association with HBV integration in hepatocellular carcinoma were studied. HBV integration was accompanied by a deletion of at least 13.5 kilobases of cellular sequences in a human hepatocellular carcinoma. The viral DNA integration and deletion of cellular sequences occurred on a short arm of chromosome 11 in location 11P13 to 11P14. The cellular sequences that were deleted at the site of HBV integration were lost from the tumor cell, leaving only a single copy of the remaining cellular allele.

Oncogenic viruses, in general, cause a variety of chromosomal aberrations; and deletions at viral integration sites are not unique to HBV. Both SV40 and Epstein-Barr virus DNA integration caused deletions of up to 15 kilobases of cellular DNA in transformed cells. Even though the function of these deletions is currently unknown, the high correlation between chromosomal abnormalities and carcinogenesis supports the idea that deletions may be a part of a multistep mechanism leading to cell transformation. Therefore, the ability of HBV integrations to mediate deletions and other chromosomal aberrations may be related to the oncogenic potential of this virus (70).

A long-term study on the causes of morbidity and mortality in duck hepatitis B virus (DHBV)-infected and uninfected flocks of Pekin ducks is focused on determining if more liver disease is present in the virus-infected flock. Necropsies and histological examinations were performed on four virus-infected and five uninfected ducks this year. Only one duck, a DHBV-positive animal, exhibited significant hepatitis. As in the past years, no tumors were observed, with the major cause of death being amyloid disease. Parallel studies with hepadnavirus-infected ground squirrels have shown that hepatocellular carcinoma develops in animals with virus markers late in the squirrel's life span rather than in the young to middle years as in hepadnavirus-infected woodchucks. A similar late development of liver tumors in animals not exposed to other carcinogens may also occur in the Pekin duck system.

Previous studies by Robinson showed that one-week-old chicks were not susceptible to duck hepatitis B virus infection when injected with an inoculum causing duck hepatitis viremia in ducklings. To further probe the host specificity of this virus, 12 one-week-old Muscovy ducklings and 12 one-week-old Embden goslings were injected with infectious DHBV. Like Pekins, Muscovy ducks are

members of the Anapidae sub-family Anapinae, while the domestic geese, like the Embden breed, are classified in the subfamily Anserinae. No virus was detected in sera from any of the birds prior to infection. The birds were bled weekly and the Muskovys and half the geese sacrificed at six weeks. All sera and DNA extracted from the livers were tested for DHBV DNA sequences by slotblot DNA hybridization. All samples from the Muskovy ducks were negative for DHBV DNA. In contrast, 11 out of 12 of the more distantly related geese became highly viremic one to three weeks following DHBV injection, with four of the five unsacrificed viremic animals becoming chronic carriers (viremic for six months). In this experiment and in a larger repeat experiment using injected Pekins and uninjected Embden geese as additional controls, a different pattern of infections was noted in the geese compared to the ducks. While virus titer in injected ducklings was highest one week after injection, the titer in infected geese rose gradually to the highest at six to seven weeks, suggesting differences in host susceptibility or immune response in the two species.

In other studies it was demonstrated that young, virus-infected ducklings whose virus titer is generally higher than adults and whose maintenance costs are less than other models can be used to test anti-virals to hepadnaviruses provided that the anti-viral is not metabolized differently in birds than in mammals (as appears to be the case with adenine arabinoside) (69).

In spite of a number of significant observations, the central questions of how oncogenes function remain unanswered. Some preliminary answers are beginning to emerge on the function of oncogene proteins in cells (growth factor receptors, etc.) but further work is necessary to determine whether or not these preliminary studies will be verified. Additional activities are also ongoing in studies of the hepadnaviruses, with the finding that in some cases ducklings (which are less expensive to maintain than other animal models, such as chimpanzees) can be used to test anti-virals proposed for use in treating hepatitis infections.

RNA VIRUS STUDIES II
GRANTS ACTIVE DURING FY86

<u>Investigator/Institution/Grant Number</u>	<u>Title</u>
1. ACS, George Mount Sinai School of Medicine 5 R01 CA 34818-03	Studies on the Replication and Oncogenicity of HBV
2. BALDUZZI, Piero C. University of Rochester 2 R01 CA 32310-04	The Transforming Genes of Avian RNA Tumor Viruses
3. BALUDA, Marcel A. University of California (Los Angeles) 2 R01 CA 10197-19	Tumor Induction by Avian Myeloblastosis Virus
4. BEEMON, Karen L. Johns Hopkins University 2 R01 CA 33199-04	Location and Function of m6A in Retrovirus RNAs
5. BISHOP, J. Michael University of California (San Francisco) 5 R01 CA 12705-15	Rous Sarcoma Virus: Replication and Cell Transformation
6. BOETTIGER, David E. University of Pennsylvania 5 R01 CA 16502-12	Genetic Analysis of RNA Tumor Viruses
7. BOETTIGER, David E. University of Pennsylvania 2 R01 CA 30383-06	Virus-Induced Myeloid Leukemia
8. BOSE, Henry R., Jr. University of Texas (Austin) 5 R01 CA 33192-03	Transformation by Avian Reticu- loendotheliosis Virus
9. BOYD, Juanell N. Cornell University (Ithaca) 5 R23 CA 36160-03	Dietary Choline, Aflatoxin, and Carcinogenesis
10. BRUGGE, Joan S. State University of New York (Stony Brook) 5 R01 CA 27951-07	The Association of Two Cellular Proteins with pp60- <u>src</u>
11. BUSS, Janice E. Salk Institute for Biological Studies (San Diego) 1 R23 CA 42348-01	Attachment of Myristic Acid to P60- <u>src</u>

12. BUTEL, Janet S.
Baylor College of Medicine
5 R01 CA 37257-03
Hepatitis B Virus and Human Liver
Cancer
13. CARBON, John A.
University of California
(Santa Barbara)
5 R01 CA 11034-18
Studies on Gene Organization and
Expression
14. CASPÄR, Donald L.
Brandeis University
5 R01 CA 15468-13
Assembly of Viruses, Membranes,
and Tissue
15. CHAKRABORTY, Prasanta R.
Medical College of
Pennsylvania
5 R01 CA 38007-02
Gene Expression--Oncogenic/
Chronic Hepatitis B
Infection
16. CHEN, Ji Hsiung
St. Jude Children's
Research Hospital
1 R01 CA 42859-01
The Transforming Gene of Avian
Acute Leukemia Virus E2
17. CHISARI, Francis V.
Scripps Clinic and Research
Foundation
5 R01 CA 40489-02
Pathogenesis of Hepatitis B
18. CHRISTENSEN, James R.
University of Rochester
5 R01 CA 36312-03
Oncogenes and Neoplastic
Progression
19. COFFIN, John M.
Tufts University
5 R01 CA 17659-11
Relationship of Avian Tumor Virus
RNA and Host Genome
20. COFFIN, John M.
Tufts University
5 R01 CA 27108-07
Mechanisms of Variability of
Tumor Virus RNA
21. COOPER, Jonathan A.
Fred Hutchinson Cancer
Research Center
1 R01 CA 41072-01A1
Protein Phosphorylation and Cell
Growth Regulation
22. DUESBERG, Peter H.
University of California
(Berkeley)
2 R01 CA 11426-17
Retroviral Onc Genes and Cellular
Proto-Onc Genes
23. DUESBERG, Peter H.
University of California
(Berkeley)
5 R35 CA 39915-02
Retroviral Onc Genes and Cellular
Proto-Onc Genes

24. EISENMAN, Robert N.
Fred Hutchinson Cancer
Research Center
5 R01 CA 20525-10
Control Mechanisms in Avian
Oncornavirus Replication
25. ERIKSON, Raymond L.
Harvard University
5 R01 CA 34943-05
Biosynthesis of Viral RNA
26. FARAS, Anthony J.
University of Minnesota
(Minneapolis-St. Paul)
5 R01 CA 18303-12
RNA-Directed DNA Polymerase and
70S RNA of Oncornaviruses
27. GOLDBERG, Allan R.
Rockefeller University
5 R01 CA 13362-15
RSV Functions Involved in
Transformation
28. GORDON, Julius A.
University of Colorado Health
Sciences Center
5 R01 CA 35378-03
Studies of pp60-src Activity and
Substrate Phosphorylation
29. GOULIAN, Mehran
University of California
(San Diego)
5 R01 CA 11705-17
DNA Synthesis Studies
30. GRANDGENETT, Duane P.
St. Louis University
5 R01 CA 16312-13
Avian Retrovirus DNA Synthesis
and Its Regulation
31. GRANOFF, Allan
St. Jude Children's Research
Hospital
5 R01 CA 07055-24
Studies on Lucke Tumor Associ-
ated Viruses
32. GRODZIKER, Terri I.
Cold Spring Harbor Laboratory
1 R13 AI/CA 22056-01
Hepatitis B Virus Meeting
33. GUNTAKA, Ramareddy V.
University of Missouri
(Columbia)
5 R01 CA 36790-04
Synthesis and Structure of Avian
Tumor Virus DNA
34. HALPERN, Michael S.
Wistar Institute of Anatomy and
Biology
2 R01 CA 31514-05
Endogenous Retrovirus as a
Determinant of Tumor Immunity
35. HANAFUSA, Hidesaburo
Rockefeller University
2 R01 CA 14935-14
Cellular Alteration Induced by
Rous Sarcoma Virus

36. HARRISON, Stephen C.
Harvard University
5 R01 CA 13202-15
Structure and Assembly of Viruses
and of Coated Vesicles
37. HAYWARD, William S.
Sloan-Kettering Institute for
Cancer Research
5 R01 CA 34502-05
RNA Tumor Virus Gene Expression
38. HAYWARD, William S.
Sloan-Kettering Institute for
Cancer Research
1 R01 CA 43250-01
Mechanisms of Viral and Non-
viral Oncogenesis
39. HOLTZER, Howard
University of Pennsylvania
5 R01 CA 18194-10
Conversion of Embryonic Cells
Into Transformed Cells
40. HUMPHRIES, Eric H.
University of Texas Health
Science Center (Dallas)
2 R01 CA 32295-04
Characterization of the ALV-
Induced Transformed Follicle
41. HUMPHRIES, Eric H.
University of Texas Health
Science Center (Dallas)
1 R01 CA 41450-01
Expression and Function of v-rel
in Lymphoid Tissue
42. HUNTER, Eric
University of Alabama
(Birmingham)
5 R01 CA 29884-06
Site Specific Mutagenesis of the
Envelope Gene of RSV
43. JOKLIK, Wolfgang K.
Duke University
5 P01 CA 30246-06
Regulatory Functions of Protein
Nucleic Acid Interaction
44. KNOWLES, Barbara B.
Wistar Institute of Anatomy and
Biology
5 R01 CA 37225-03
Hepatitis B Virus and Primary
Hepatocellular Carcinoma
45. KOPROWSKI, Hilary
Wistar Institute of Anatomy and
Biology
5 P01 CA 21124-09
Genetics and Virology of Cancer
46. KUNG, Hsing-Jien
Case Western Reserve University
5 R01 CA 38659-02
Oncogene and Activator:
Tumorigenesis by Cloned DNA
47. KUNG, Hsing-Jien
Case Western Reserve University
2 R01 CA 39207-02
Avian Erythroleukemia and
c-erb-B Activation

48. LAU, Alan F.
University of Hawaii (Manoa)
5 R01 CA 35578-03
Cellular Substrates of pp60-src
in ASV-Infected Cells
49. LEE, Wen-Hwa
University of California
(San Diego)
5 R01 CA 39537-02
Transforming Gene and Protein
of Fujinama Sarcoma Virus
50. LEIS, Jonathan P.
Case Western Reserve University
5 R01 CA 38046-03
Studies of Retroviral Proteins
51. LINIAL, Maxine L.
Fred Hutchinson Cancer Research
Center
5 R01 CA 18282-11
Viral Coded Functions in Rous
Sarcoma Virus
52. MACARA, Ian G.
University of Rochester
5 R01 CA 38888-02
Oncogene Phosphoinositide Kinase
Activity and Cancer
53. MAJORS, John E.
Washington University
5 R01 CA 38994-02
Analysis of Retroviral Tran-
scriptional Regulation
54. MARTIN, G. Steven
University of California
(Berkeley)
5 R01 CA 17542-11
Genetics of RNA Tumor Viruses
55. MILLER, Arthur D.
Fred Hutchinson Cancer Research
Center
1 R01 CA 41455-01
Gene Transfer Using Retroviral
Vectors
56. MONTELARO, Ronald C.
Louisiana State University
(Baton Rouge)
5 R01 CA 38851-06
EIAV: Antigenic Variation and
Retrovirus Persistence
57. MOSCOVICI, Carlo
University of Florida
2 R01 CA 10697-20
Avian Leukemia Viruses and Cell
Differentiation
58. NEIMAN, Paul E.
Fred Hutchinson Cancer
Research Center
2 R01 CA 20068-11
Molecular Mechanisms in Neoplasia
59. NEIMAN, Paul E.
Fred Hutchinson Cancer
Research Center
2 P01 CA 28151-07
Program in Retroviruses and
Cancer

60. OGSTON, Charles W.
Rush-Presbyterian-St. Luke's
Medical Center
5 R01 CA 37276-03
Molecular Biology of Hepatitis
Virus In Vivo
61. PARSONS, John T.
University of Virginia
(Charlottesville)
5 R01 CA 27578-06
Expression of Avian Retrovirus
Transforming Genes
62. PARSONS, John T.
University of Virginia
(Charlottesville)
5 R01 CA 29243-06
Avian Sarcoma Virus-Specific
Tumor Antigens
63. PARSONS, Sarah J.
University of Virginia
(Charlottesville)
5 R01 CA 39438-02
Role of c-src in Retroviral
Transformation
64. PERDUE, Michael L.
University of Kentucky
(Lexington)
1 R01 CA 39554-01A2
Regulation of Protein Synthesis
by Retrovirus Leader
65. PRIVALSKY, Martin L.
University of California (Davis)
5 R01 CA 38823-02
Characterization of the v-erb-B
Oncogene Protein of AEV
66. RHODE, Solon L., III
University of Nebraska Medical
Center
5 R01 CA 37481-02
Replicon Control in Normal and
Transforming Cells
67. ROBINSON, Harriet L.
Worcester Foundation for
Experimental Biology
5 R01 CA 23086-09
Retrovirus and Congenital
Transmission
68. ROBINSON, Harriet L.
Worcester Foundation for
Experimental Biology
5 R01 CA 27223-07
Avian Leukosis Viruses and Cancer
69. ROBINSON, William S.
Stanford University
5 R01 CA 34514-04
Duck Hepatitis B Virus:
Infection and Disease
70. ROGLER, Charles
Yeshiva University
5 R01 CA 37232-03
Molecular Aspects of WHV-Induced
Persistent Infection

71. ROHRSCHEIDER, Larry R.
Fred Hutchinson Cancer
Research Center
5 R01 CA 20551-10
Mechanisms of Oncornavirus-
Induced Transformation
72. RUECKERT, Roland R.
University of Wisconsin (Madison)
5 R01 CA 08662-19
Structure and Synthesis of Retro-
and Nodaviruses
73. SCHUBACH, William H.
State University of New York
(Stony Brook)
7 R23 CA 42168-01
Structure and Expression of the
Endogenous myc Region
74. SCOTT, June R.
Emory University
5 R01 CA 11673-16
Lysogeny and Bacteriophage P1
75. SEFTON, Bartholomew M.
Salk Institute for Biological
Studies
5 R01 CA 17289-11
Viral Membranes and Viral
Transformation
76. SHAFRITZ, David A.
Yeshiva University
5 R01 CA 32605-05
Hepatitis B Virus - Chronic
Hepatitis - Liver Cancer
77. SHALLOWAY, David I.
Pennsylvania State University
(University Park)
5 R01 CA 32317-05
Role of pp60c-src Homolog of the
RSV Oncogene Protein
78. SHANK, Peter R.
Brown University
5 R01 CA 32980-05
Stability and Disease Tropism of
Proviral DNAs
79. SHENK, Thomas E.
Princeton University
5 R01 CA 39606-02
Functional Analysis of the
Adeno-Associated Virus Genome
80. SMITH, Ralph E.
Colorado State University
2 R01 CA 35984-04
Biochemistry of RNA Tumor Virus
Replication
81. STAVNEZER, Edward
University of Cincinnati
1 R01 CA 43600-01
Origin Structure and Biological
Activity of SKVS
82. STOLTZFUS, Conrad M.
University of Iowa
5 R01 CA 28051-07
Retrovirus RNA Metabolism

83. SUMMERS, Jesse W.
Institute of Cancer Research
(Philadelphia)
1 R35 CA 42542-01
Persistent Infections by Hepadna-
viruses
84. SWANSTROM, Ronald I.
University of North Carolina
(Chapel Hill)
5 R01 CA 33147-03
Retrovirus Replication: Inter-
action with Host Genome
85. TATTERSALL, Peter J.
Yale University
5 R01 CA 29303-06
Molecular Basis of Parvovirus
Target Cell Specificity
86. TAYLOR, John M.
Institute for Cancer Research
(Philadelphia)
5 R01 CA 22651-09
Reverse Transcription
87. TEMIN, Howard M.
University of Wisconsin (Madison)
5 P01 CA 22443-09
Molecular Biology and Genetics
of Tumor Viruses
88. TENNANT, Bud C.
Cornell University (Ithaca)
5 R01 CA 37264-03
Hepatitis, Aflatoxin, and
Hepatocarcinogenesis
89. TIOLLAIS, Pierre
Pasteur Institute
5 R01 CA 37300-03
Hepatitis B Virus DNA, Oncogenes,
and Liver Cancer
90. VARMUS, Harold E.
University of California
(San Francisco)
5 R01 CA 37281-03
Oncogenic Potential of the
Hepatitis B-Type Viruses
91. VARMUS, Harold E.
University of California
(San Francisco)
5 R35 CA 39832-02
Molecular Analysis of Retro-
viruses and Oncogenes
92. VOGT, Peter K.
University of Southern California
5 R01 CA 13213-15
Interactions Between Avian Tumor
Viruses and Their Hosts
93. VOGT, Peter K.
University of Southern California
5 R01 CA 29777-05
Avian Oncoviruses: Transforming
Genes and Proteins
94. VOGT, Peter K.
University of Southern California
1 R35 CA 42564-01
Onc Genes in Virus and Cell

95. VOGT, Volker M.
Cornell University (Ithaca)
5 R01 CA 20081-10
Avian Retrovirus Structure and
Assembly
96. WANDS, Jack R.
Massachusetts General
Hospital
5 R01 CA 35711-03
Pathogenesis, Immunodiagnosis,
and Therapy of Carcinoma
97. WANG, Lu-Hai
Rockefeller University
5 R01 CA 29339-06
Transforming Genes of Avian
Sarcoma Viruses
98. WEBER, Michael J.
University of Virginia
(Charlottesville)
5 R01 CA 39076-03
Early Cellular Changes in Viral
Oncogenesis
99. WEINTRAUB, Harold
Fred Hutchinson Cancer
Research Center
1 R35 CA 42506-01
Generation of Development Mutants
with Cloned DNA Vectors

SUMMARY REPORT
RESEARCH RESOURCES

The Research Resources component of the Biological Carcinogenesis Branch (BCB), in conjunction with the various research units of the Branch, is responsible for developing, allocating and maintaining a coordinated program of research material support to meet the needs of extramural investigators funded by the Branch. The planning, initiating, and oversight necessary to generate and maintain specific research resources is the responsibility of the individual Program Directors who administer each of the research components of the Branch. However, the storage and distribution of research materials, the management of some resource contracts, the development and maintenance of a computerized inventory, and the day-to-day general management and direction of all resources distribution are the responsibility of this component of the Branch. There are currently eight research resources contracts with an estimated funding level of 2.1 million dollars. One new resource contract for operation of the repository for storage and distribution of biological reagents was awarded during this period.

Laboratory investigations carried out under the sponsorship of the BCB depend on the availability of adequate quantities of viruses, viral reagents, antisera, animals and clinical and laboratory materials of adequate purity, viability and potency, some of which are not available from the commercial sector. The BCB resources component provides some research materials and other supporting activities through contract operations in four general areas. These include activities directed toward production, characterization and distribution of viral and anti-viral reagents; activities concerned with animal resources, including breeding and maintenance of animal colonies; activities directed toward the provision of specialized testing services for the examination of experimental materials; and activities concerned with the storage, inventory and distribution of human specimens.

The viral reagents produced during this period to meet program needs included avian myeloblastosis virus (AMV) reverse transcriptase and monoclonal antibodies to oncogene products of retroviruses. A consistently active supply of AMV reverse transcriptase is vital to biological carcinogenesis studies involving the production of cDNA copies of retrovirus genomes for use as probes to identify viral sequences in normal or malignant tissues, to compare viral and cellular sequences for homology, to permit expression of viral sequences in bacterial systems and for other molecular biological studies. To meet these needs, more than 525,000 units of AMV reverse transcriptase were produced and 150 shipments were made to domestic and foreign laboratories. The production and distribution of monoclonal antibodies to oncogene products and the synthetic peptides representing determinants of various regions of the oncogene products continued during this period with approximately 250 items of monoclonal reagents being shipped to more than 40 laboratories (4,6).

Animals have an important role in the biological carcinogenesis research program. Studies to determine the biological activity of putative human cancer viruses cannot be carried out in humans; therefore, it is imperative that another system be developed for these studies and subsequently for the evaluation of vaccines or other measures of control. Since the marmoset appears

to be especially suitable for use as a comparative model system, the Branch supports the operation and maintenance of a breeding colony for marmosets. The colony currently contains 260 tamarins and marmosets. One collaborative study has been established to study marmoset colitis and colon cancer which appears to be an excellent model system for human colon cancer. At the end of fiscal year 1985, a resource contract was initiated for the maintenance of a herd of cattle and sheep infected with bovine leukemia virus, along with uninfected control animals, to provide research investigators with blood, lymphocytes, plasma and bone marrow smears for virus cancer research. Since cost recovery for the materials provided by each of these contracts has, thus far, been minimal, close evaluation of need for and use of these reagents will be continued. If use of the materials supplied by these contractors remains at a low level, these efforts will be reduced or terminated (1,3).

Additionally, a transfer of funds in the amount of \$385,000 was made to the National Institute of Allergy and Infectious Diseases to support production of captive-born woodchucks for utilization as models for studies of human hepatitis and hepatocellular carcinoma. Significantly, it appears that the chronic carrier state can be established in the woodchuck. This animal may furnish a valuable model for studies on the development of hepatocellular carcinoma in humans; moreover, it is apparently superior to the duck model in that histopathologic characteristics of hepatoma have been demonstrated in the woodchuck and not, as yet, in the duck. These animals are being used in experimental studies of hepatocellular carcinoma by NCI grantees and the breeding effort to produce captive bred seronegative woodchucks will continue (8).

In the search for oncogenic viruses, many cell cultures from the same or different species are used concurrently, thus offering frequent opportunities for cross-contamination. In cross-species tumor transplantations, it is important to be able to determine the derivation of induced tumors. Additionally, the significance of the presence of virus in tissue cells, the ability to grow virus, or the validity of virus isolation systems are all dependent upon the assurance of the identity of the cell cultures used. To meet this need, procedures were carried out for interspecies and intraspecies cell identification on approximately 275 cultures from 44 laboratories. Three assays were utilized in these determinations: immunofluorescent staining for species specific surface antigens, isoenzyme analysis, and cytological analysis by means of chromosome banding (7).

During this period, viral reagents and human specimens from the inventory of frozen biological reagents, along with appropriate demographic, clinical and characterization data, were shipped to more than 60 domestic and foreign laboratories (2,5).

The Branch has been involved in implementation of the resources "payback" system since 1981. The payback system is one in which the recipients of resource materials or services reimburse the resource or production contractor directly for the services or materials received, based on a price schedule agreed upon in advance between the NCI and its resource contractor. The contractor in turn credits those funds received from recipients against its production costs and these are shown on monthly vouchers which it submits to the Government for payments under the contract. Initiation of this system was the result of a

variety of influences: the noticeable shrinking of the budget; an interest in seeing that the resource dollars utilized by grantees, intramural scientists, and contractors were included in a peer-review system; and the perception that free distribution of resources did not always result in the most effective utilization of available funds.

All resource contracts operate under the payback system. Total costs of production and distribution of research materials are collected on the contracts in which there are a large number of individual users who are receiving small amounts of material at costs reasonable enough for them to continue to acquire them without financial hardship. In other cases, where past utilization patterns have shown that significant problems would be encountered if total costs were charged, only partial operating costs are collected in order that investigators will not have to unduly curtail their research activities. In either case, as a general rule, all grantees, contractors, and intramural scientists pay for the resources which they receive. The payback system overall seems to be performing as expected. The demand level for high quality biological reagents not readily available from commercial sources has remained fairly constant. Reimbursement for full or partial costs of services has led to more careful use of costly resource reagents. This has resulted in a reduced level of effort in several resource contracts or the termination of now unnecessary activities.

RESEARCH RESOURCES

CONTRACTS ACTIVE DURING FY86

<u>Investigator/Institution/Contract Number</u>	<u>Title</u>
1. CLAPP, Neal K. Oak Ridge Associated Universities NO1 CP 51006	Operation of a Marmoset Colony for Cancer Research
2. CLARKE, Jane D. Microbiological Associates NO1 CP 61020	Repository for Storage and Distribution of Viruses, Sera, Reagents and Tissue Specimens
3. FERRER, Jorge F. University of Pennsylvania NO1 CP 51003	Bovine Leukemia Virus Herd
4. HOUTS, G. E. Molecular Genetics Resources, Inc. NO1 CP 51007	Production, Characterization and Distribution of AMV Reverse Transcriptase
5. MASSAGEE, Pamela D. Microbiological Associates NO1 CP 11000	Repository for Storage and Distribution of Viruses, Sera, Reagents and Tissue Specimens
6. NIMAN, Henry L. Scripps Clinic and Research Foundation NO1 CP 41009	Preparation of Monoclonal Anti- bodies to Oncogene Products of Retroviruses
7. PETERSON, Ward D. Children's Hospital of Michigan (Detroit) NO1 CP 21017	Inter- and Intraspecies Identification of Cell Cultures
8. TENNANT, B. C. Cornell University (Ithaca) NO1 AI 52585	Breeding Facility for Woodchucks (Marmota Monax)





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